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Nitrogen and Warming Effects on Nitrous Oxide Production Associated with Coastal Macroinvertibrates

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NITROGEN AND WARMING EFFECTS ON NITROUS OXIDE PRODUCTION ASSOCIATED WITH COASTAL MACROINVERTEBRATES

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ABSTRACT

Bivalve shellfish potentially reduce excess nitrogen in the water column, however they can also be involved in the emission of nitrous oxide (N_2O), a potent greenhouse gas. Environmental controls on N_2O production from bivalves have not been well quantified. We tested responses of N_2O production by three bivalves (*Mytilus edulis, Mercenaria mercenaria* and *Crassostrea virginica)* to nitrogen (N) loading and/or warming after immediate (1 day) and short-term (14-28 days) exposure. This two-factor laboratory study had four treatments: (1) ammonium nitrate (N) addition (targeting 100μM-N), (2) warming (22^{o}C), (3) N addition + warming and (4) no N addition or warming (control, 19^{o}C). Potential N_2O production rates were higher in response to N additions for all bivalves, particularly with short-term exposures. Warming had a small but significant impact on N_2O production from *M. mercenaria,* confounded by a significant interaction of exposure X warming and exposure X nitrogen X warming. Similarly, *C. virginica* also showed a significant interaction of exposure X warming, indicating that longer exposure to warming may influence N_2O production from this species. *M. edulis* showed the highest N_2O production rates, reaching 252 nmol N_2O ind^{-1} hr^{-1}, more than an order of magnitude higher than the previously largest reported rates. However, mass-specific rates (7.5 nmol N_2O g^{-1} hr^{-1}) were the same order of magnitude as previous studies. Notably, N_2O production associated with *M. edulis* were obtained while the organisms had poor health, which likely induced high respiration rates and was probably caused by hypoxic water conditions. We also examined the influence of macro-epifauna on the N_2O production associated with *M. edulis* via removal of macro-epifauna. There was no significant

difference in N_2O produced by *M. edulis* with and without epifauna, which suggests that N_2O production may be largely due to gut microbial activity and microbial biofilms on the shells from *M. edulis*. In summary, our study indicates a strong influence of N on the potential N_2O emissions rates of prominent bivalves, which should be considered when incorporating them into coastal N mitigation strategies.

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INTRODUCTION

Bivalves are frequently used in water quality restoration projects, as they can mitigate the negative consequences of eutrophication by significantly impacting fixed nitrogen (N) cycling in aquatic systems (Stief 2013; Kellogg et al. 2014). Through filter-feeding, they can influence benthic-pelagic coupling by removing fixed N and phytoplankton biomass from the water column that together contribute to eutrophic systems (Carmichael et al. 2012; Kellogg et al. 2014). Along with fixed N, bivalves ingest denitrifiers that can remain metabolically active in their anoxic gut micro-environment, which further impacts aquatic N cycling (Stief et al. 2009). Also, reef building bivalves can enhance the surface area for N-cycling microbes to grow on their shells, thereby removing or producing N through nitrification, denitrification or coupled processes (Svenningsen et al. 2012; Heisterkamp et al. 2013). Bivalves can also be sources of ammonia through their excretions and biodeposits at high rates such that this may counter the amount of fixed N they can remove from the water column (Stief 2013). Importantly, recent investigations have found that by enhancing N-cycling processes, bivalves can also stimulate the production of nitrous oxide (N_2O) (Stief et al. 2009; Heisterkamp et al. 2010; Svenningsen et al. 2012), a potent greenhouse gas that is a major contributor to climate change and ozone depletion (Forster et al. 2007).

Denitrification in the anoxic guts of bivalves as well nitrification and/or denitrification on their shell biofilms produce significant amounts of N_2O to the water column (Svenningsen et al. 2012; Heisterkamp et al. 2013). N_2O is produced through microbial nitrification (the oxidation of ammonium to nitrate) during the first

oxidation step (Goreau et al. 1980) and as a byproduct in denitrification and nitrifier denitrification (Zumft 1997; Wrage et al. 2001). When complete, denitrification is actually a sink for N_2O (converting it to N_2), but in the case of invertebrate guts, the pathway may be terminated early with N_2O due to inhibition of N_2O reductase (*nosZ*) by excess nitrate similar to marine sediments (Miller et al. 1986). The sources of N_2O production can vary among species as Svennignsen et al. (2012) found that approximately 25% of the N_2O production associated with the zebra mussel, *Dreissena polymorpha* was ascribed to the shell biofilm, while Heisterkamp et al. (2013) found that the N_2O production from the blue mussel, *Mytilus edulis,* was almost entirely due to shell biofilm microbiota. N_2O production from invertebrates is also known to be positively related to biomass (Stief and Eller 2006; Stief et al. 2009; Stief and Schramm 2010), whereby large bivalves can have large N_2O production potentials compared to smaller individuals. This is due to the increase in gut size with increase in biomass and can therefore house more microbial consortia. Bivalve associated N_2O production is of particular concern due to their large abundances in benthic coastal habitats and in aquaculture (Stief et al. 2009; Svenningsen et al. 2012; Heisterkamp et al. 2013); thus, these areas can be potential "hot spots" of N_2O emissions to the atmosphere.

Excess fixed N and ocean warming are key environmental stressors that may increase N_2O emissions, though the effects on marine shellfish have been largely understudied. The increased availability of fixed N fertilizer and atmospheric deposition is affecting coastal habitats worldwide (Gruber and Galloway 2008). N_2O is known to be emitted in these areas of high concentrations of fixed N in sediments

and water column (Seitzinger et al. 1983; Kroeze and Seitzinger 1998; Corredor et al. 1999; Muñoz-Hincapié et al. 2002). Thus, highly eutrophic habitats may significantly increase N_2O production associated with marine bivalves by providing copious amounts of substrate for nitrification, denitrification and coupled processes to occur. Also, N_2O production rates can increase as a function of temperature due to higher microbial activities as has been shown in marine sediments, grasslands and in microbial biofilms in rivers (Kroeze and Seitzinger 1998; Boulêtreau et al. 2012). Consequently, ocean warming may stimulate N_2O production by increasing the metabolism of nitrifying and denitrifying bacteria on the shells and within the guts of bivalves. Ocean warming, driven by enhanced carbon dioxide in the water, can significantly decrease growth of larvae and juvenile bivalves, increase mortality and increase respiration (Dove and Sammut 2007; Dickinson et al. 2012; Matoo et al. 2013; Mackenzie et al. 2014). Impaired health of the organism due to warming may stimulate more oxygen demand and thus increase filter-feeding, ingestion of denitrifiers and subsequent N_2O emissions. Further, filtration rates and temperature have a positive relationship (Kittner 2005), which would potentially increase fixed N into bivalve guts, stimulating denitrification. Thus, synergistic interactions between N rich environments and warming can potentially intensify N_2O production associated with marine bivalves.

The goal of this research was to: (1) examine the effect of N addition and/or warming on N_2O production from three coastal bivalves (*Mytilus edulis*, *Mercenaria mercenaria* and *Crassostrea virginica),* (2) determine if biomass is a control for N_2O production and (3) examine the contribution of macro-epifauna on one invertebrate

(*M. edulis*) to overall N_2O production rates via a macro-epifauna-removal experiment in the presence and absence of N loading. In order to assess how elevated N and/or warming affects rates of N_2O production from these taxa, a two-factor aquarium study with 4 treatments was used: (1) N addition, (2) warming, (3) N addition + warming and (4) controls (with no N addition or warming). N_2O production rates were examined after an immediate (1 day) exposure and short-term (14-28 day) exposures. We hypothesized that both N additions and warming treatments would significantly increase the N_2O emissions rates from each species and that N_2O would increase between exposure periods. We also expected *M. mercenaria* to have the highest N_2O production per gram of biomass and the smallest species, *C. virginica* to emit the lowest amount per gram. Lastly, we anticipated macro-epifauna organisms to contribute significantly to the N_2O production of *M. edulis,* particularly in the presence of N additions. To identify further potential controls on N_2O emissions by these invertebrates, we tested relationships of N_2O production rates to water quality parameters (pH, DO, dissolved inorganic nitrogen) and physiological status of the three bivalve species. Our study contributes to the few studies that have examined bivalve N_2O production, especially those in response to global change drivers.

METHODS

Field site and collection

For this study, multiple individuals of *M. edulis, M. mercenaria* and *C. virginica* were collected from subtidal Narragansett Bay, Rhode Island, USA through scuba diving in Narragansett, Conimicut Point and North Kingstown, respectively. *C.*

virginica was collected during the early summer season. *M. mercenaria* and *M. edulis* were collected during the late summer.

Narragansett Bay, Rhode Island has a well-documented bay-wide gradient in anthropogenic N loads, declining from the north to south (Deacutis 2008; Oczkowski et al. 2008). The nutrient maximum within the bay is in the Providence River and upper bay, with an annual average total nitrogen concentrations (TN) of l70 µM and total phosphorus (TP) of 5 µM, including dissolved particulate, organic and inorganic forms (Oviatt et al. 2002). The lowest nutrient levels are in Rhode Island Sound with an annual average concentrations for TN of 12 µM and TP of 1 µM (Oviatt et al. 2002). There is minimal freshwater input into the bay, thus surface and bottom salinities are high, ranging from 20-32 ppt (Pilson 1985). Water temperatures of the Narragansett Bay range from 1 ºC to 23 ºC in the middle and lower bay with minimal differences between surface and bottom waters (Kremer and Nixon 1979). Warmer waters have been observed over recent decades, with an average 2.2ºC increase during the winter since 1960 (Nixon et al. 2009), along with occasional hypoxic events (Deacutis et al. 2006; Codiga et al. 2009).

We collected seawater (40 mL) at the surface and at the depth of each bivalve collection site (depths ranging from 3-8 meters) and analyzed them on board the research vessel to determine salinity using a handheld refractometer, temperature, pH and dissolved oxygen (DO, Thermo Scientific Orion Star A326 pH/Dissolved Oxygen Portable Multiparameter Meter). To determine dissolved inorganic nitrogen (DIN) concentrations, a subsample of the water was filtered using disposable syringe filters (Advantec; 0.45 µm; sterile) and frozen (-17ºC) at the time of collection and later

analyzed for ammonia and nitrate using a micro-segmented continuous flow nutrient analyzer (Astoria Analyzer, model 303a).

Immediately after collection, organisms were transported to environmental temperature chambers (Holman Engineering) at the Marine Sciences Research Facility (MSRF) at the University of Rhode Island Bay Campus. They were acclimated in clear glass "batch style" aquaria (Figure 1) in aerated, unfiltered seawater at either ambient water temperature (19ºC) or warming treatment (22ºC) for at least 12 hours before start of the incubations. Gas tight tops for the aquaria were equipped with inflow and outflow sampling ports (Figure 1). A magnetic stir bar inside each tank was used and gently driven at 40 rotations per minute by a magnetic carousel in order to homogenize the water (Figueiredo-Barros et al. 2009). Unfiltered seawater pumped directly from Narragansett Bay was used in order to simulate natural microbial populations and to maintain a food source similar to that *in situ*.

Nitrogen and warming manipulations

Nitrogen and warming manipulations (NxW) were applied to invertebrates with 3-4 replicate aquaria (10.1 L, Figure 1) which had been acclimating overnight in the same aquarium. For N addition treatments, each aquarium received a pulse of 10mM ammonium nitrate prepared in unfiltered seawater at a volume sufficient to increase the overall DIN concentration to 100 μM-N. This N level was selected in order to target maximal total N concentrations in the bay that were previously reported (Oviatt et al. 2002). N additions were made to the same water that the invertebrates had been acclimating in and immediately prior to sealing the aquarium for each of the five-hour incubations. All aquaria were maintained in the dark (except during

sampling and tank maintenance) to limit DIN uptake by any active phytoplankton. Based on approximate field abundances in New England waters (Schulte et al. 2009; Tam and Scrosati 2011), three individuals were added per aquarium for *M. edulis* and *C. virginica*. *M. mercenaria* aquaria received 1 individual per tank (Thelen and Thiet 2009). Aquaria were randomly assigned to treatments. Seawater was replaced (with appropriate N levels and water temperatures) every other day in order to maintain adequate food supply for the organisms and in attempt to control ammonia accumulation from animal excretions.

Temperatures were manipulated by placing the aquaria into one of two environmentally controlled chambers. Control treatments were kept at 19^{o}C based on average summer temperatures in the bay over the past decade (Nixon et al. 2009). Since the temperature of the Narragansett Bay generally does not vary significantly between the surface and bottom during the summer months, aquaria receiving the warming treatment were maintained at 22ºC based on the average projected increase of ocean surface temperatures in New England by 2100 (Mora et al. 2013).

N_2O production rates were determined for bivalve in each aquaria over immediate (1-day) five hour long incubations in each of the four treatments. To compare responses over longer terms, the N_2O production rates were determined again after 14-days (for *M. edulis*) and 28-days (for *M. mercenaria* and *C. virginica*). These time periods will hereafter be referred to as "immediate" and "short-term", respectively. The "short-term" time period was shorter for *M. edulis* than for the other taxa due to mortality of the organisms during the course of the experiment. For *M. edulis*, two weeks was the longest time period over which at least three live organisms

could be maintained per aquarium, and three replicate aquaria were maintained rather than four for the other species. Each bivalve species was tested in a separate, sequential experiment using the same aquarium, which were cleaned with freshwater and bleach between experiments.

Sea water only experiment (no invertebrates)

To test potential N_2O production from the water column alone (without invertebrates) in the presence or absence of N additions we employed a two-factor design with the following 4 treatments: (1) filtered seawater (0.2μm), (2) filtered seawater with N addition (100 μM-N as in the bivalve experiments), (3) unfiltered seawater and (4) unfiltered seawater with N addition. Water temperatures were kept at 19ºC and followed the same protocols for the bivalve experiments as described above with a five-hour total incubation period.

Macro-epifauna contributions to M. edulis *N_2O production*

Impacts of the presence or absence of macro-epifauana on N_2O emissions from *M. edulis* were examined. This experiment focused on *M. edulis* because it had the highest N_2O emissions of the three bivalves and also the most abundant (visible) macro-epifauna, mostly composed of *Semibalanus* spp. and *Crepidula fornicata*. *M. edulis* individuals were obtained from a seawater out-take pipe at the MSRF and acclimated overnight in aquaria. Individuals were randomly assigned to each tank in a two-factor design with the following treatments: (1) no macro-epifauna (experimentally removed), (2) no macro-epifauna + N addition (at the same levels as the N addition experiment above, 100 μM-N), (3) intact macro-epifauna (not removed), (4) intact macro-epifauna + N addition. In the "no macro-epifauna"

treatments, macro-epifauna were gently removed from *M. edulis* individuals using a knife. Four *M. edulis* individuals were used per aquarium. Individuals assigned to the "macro-epifauna" treatment were handled similarly but not scraped with a knife, in attempt to maintain consistency. All aquaria for this experiment were maintained in the environmental control chambers at 19^{o}C. The duration of this experiment was the same as "immediate exposures" in the N addition and warming experiment. They were not maintained longer than one day and N_2O production was determined within five hours of the experimental manipulations.

Water samples (35mL) from the aquaria were taken with 60 mL nylon syringes equipped with stopcock valves (Cole Parmer) immediately after sealing the aquaria with a gas tight cover by first establishing a gentle siphon in the outflow sampling port, flushing out the first 35mL in the syringe with water from the aquaria, and then collecting the next 35mL of water in the syringes (T_0). The same procedure was performed for three and five hours. For aquaria with N treatments, water samples were taken after immediate N additions immediately after sealing of the lid (T_0). Samples for the *M. mercenaria* and *M. edulis* short-term N addition and warming experiments were preserved with 1mL of 50% w/v zinc chloride in gas tight septum bottles and stored at room temperature until further analysis. At time of analysis, water samples were equilibrated with ultra-high purity helium (Moseman-Valtierra et al. 2015) approximately three weeks after collection. All other samples were equilibrated within fifteen minutes of collection and headspace gas concentrations were measured on a gas chromatograph (Shimadzu GC-2014) equipped with an electron capture detector (325ºC).

N_2O production rates

For gas chromatography, helium was used as a carrier gas and a 5% methane mixture (balance argon) was used as a makeup gas with a flow rate of 2.5 mL min^{-1}. The SPL-2014 capillary column with copper packing had a flow rate was 25 mL min^{-1} based on specifications made by manufacturer (Shimadzu). Two specialty gas standards (Airgas, Billerica, MA) were used to construct standard curves with concentrations of 0.5 ppm to 2.1 ppm for N_2O. The detection limit of the GC is 0.1ppm N_2O.

The change in N_2O concentrations over this time period was approximately linear and the difference between 0 and 5 hours was used when calculating the rate of N_2O production in each aquarium. N_2O production was corrected for the dilution of the gas phase and the equilibrate distribution of N_2O between gas and water phases according to the following equation:

$$Cw = [K_0 x' P V_{wp} + (\frac{x' P V_{hs}}{RT)})/V_{wp}]$$

where C_w is the dissolved concentration of N_2O (nmol L^{-1}), K_0 is the solubility coefficient for N_2O (mol L^{-1} atm^{-1}), x' is the dry gas mole fraction of N_2O in the sample headspace (ppb), P is the atmospheric pressure (1 atm), V_{wp} is the volume of water phase (mL), V_{hs} is the volume of the headspace (mL), R is the gas constant (L atm K^{-1} mol^{-1}), and T is temperature upon equilibration (K) (Weiss and Price 1980; Walter et al. 2005).

Water quality parameters

Before and after each incubation, at 0 and 5 hours, pH and DO were measured as described above. 40 mL of water samples were also taken at the same time points, filtered (0.45 μm) and frozen for subsequent N analysis as described above.

For both experiments, changes in water properties (pH, DO) within each aquarium were tested before and after the five-hour periods of each N_2O production assay. DO was not measured for either of the *M. mercenaria* experiments or for the *M. edulis* short-term exposure due to the meter being unavailable for use. As with N_2O production rates, these were performed separately for each bivalve species.

Invertebrate condition index

After incubations, organisms were frozen (-17^{o}C) and later analyzed to determine their condition index (CI) in order to assess their physiological status. They were shucked using a blade and wet weights of soft tissues and shells were determined on an analytical balance. Organisms were then placed in a drying oven at 70°C for 48 h (when a constant weight was reached) and re-weighed. The following formula by Crosby and Gale (1990) was used to calculate the CI:

$$CI = \left[\frac{dry\ soft\ tissue\ (g)}{total\ wet\ weight\ of\ bivalve\ (g) - dry\ shell\ weight\ (g)}\right] * 1000$$

STATISTICAL ANALYSIS

A mixed model ANOVA was used to determine the statistical significance of differences in N_2O production rates (normalized per individual) among experimental treatments for N+W experiment (factor 1= nitrogen, factor 2= warming, factor 3=exposure) for each bivalve species. Due to gas loss from two syringes during

transportation, one outlier from the immediate exposure and one from the short-term exposure were removed from the *M. mercenaria* data set. N_2O production by *M. mercenaria* was log transformed to achieve equal variance among treatments. A two-factor ANOVA was used to compare biomass normalized N_2O production, mortality rates for *M. edulis* and condition indices among treatments for the three bivalve species.

Linear regressions were performed to test relationships between N_2O concentrations and water quality parameters (ammonium, nitrate, pH, DO), wet mass of the organisms and invertebrate CI. Regressions were made using all treatments and exposures per species.

A mixed model was applied to test for differences among treatments in the N addition and warming experiment for ammonium, nitrate, pH and DO.

For aquaria without invertebrates (seawater controls), differences in N_2O production rates from the water column were also tested with a two-factor ANOVA (filtration, nitrogen).

A two-factor ANOVA was used to test for significant differences in N_2O production rates by *M. edulis* in the epifauna removal experiment (epifauna presence, nitrogen).

For all ANOVAs, assumptions of equal variance and normality were tested using the Bartlett test and Shapiro-Wilk test, respectively. All statistical analyses were performed with JMP 10.0 software and significance levels of α=0.05 were used.

RESULTS

Field site characterization

Surface water ammonia (NH_4^+) concentrations ranged between 25.0- 31.5 µM-N between collection sites, while bottom water concentrations ranged from 22.6-24.4 µM-N (Table 1). Surface and bottom water nitrate (NO_3^-) concentrations ranged from 0.6-1.8 µM-N and 1.3-2.3 µM-N, respectively (Table 1). Conimicut Point had highest NH_4^+ and NO_3^- concentrations compared to Narragansett and North Kingstown collection sites, as well as lowest pH and DO concentrations (Table 1). Temperatures ranged between 12-23ºC and were generally similar between depths (Table 1). Salinity ranged 28-32 ppt and was similar among sites and depths (Table 1).

Impacts of N addition and warming on N_2O production by bivalves

N_2O production rates for *M. edulis* were 4 times greater than for *M. mercenaria* in immediate N addition exposures and 5 times greater in the short-term N addition exposures (Figures 2, 3). N_2O production rates for *M. edulis* were 10 and 32 times greater than *C. virginica* in the N addition treatments for immediate and short-term exposures, respectively (Figures 2, 4).

Mytilus edulis

M. edulis incubations under short-term exposures had the highest N_2O production rates observed in all of the experiments, averaging 251.9 ± 11.6 (+N) and 206.0 ± 47.8 (+N+W) nmol ind^{-1} hr^{-1} (Figure 2). N_2O emissions in N addition treatments were approximately five times greater in both immediate and short-term exposures than in –N-W controls, but were not impacted by warming, with no significant nitrogen X warming interaction (Figure 2, Table 2). N_2O production was approximately five times higher in the short-term exposure compared to the immediate exposure, with a significant exposure x nutrient interaction (Figure 2, Table 2).

N_2O emission rates normalized per wet mass (g) for *M. edulis* under short-term exposure (Table 3) were significantly affected by N and NW treatments, though no significant effect was seen by warming alone (Table 4).

Mercenaria mercenaria

N_2O production rates of *M. mercenaria* were significantly affected by nitrogen, warming and exposure (Figure 3, Table 2). There were also significant interactions among exposure X nitrogen, exposure X warming, and exposure X nitrogen X warming (Figure 3, Table 2). The largest change in N_2O production between immediate and short term exposures was the five-fold increase between in the N addition only treatment (Figure 3). The presence of N alone significantly affected N_2O production rates when quantified per wet mass (g) of organism for the short-term exposure treatments (Table 3,4).

Crassostrea virginica

In contrast to the other bivalves, N_2O consumption was observed in several of *C. virginica* treatments. Highest average rates of N_2O consumption were -5.5 ± 1.3 nmol N_2O ind^{-1} h^{-1} (control treatments; Figure 4). There was a significant effect of N addition on the N_2O production by *C. virginica,* and largest N_2O emissions were found in the short-term N addition only treatments (Figure 4, Table 2). There was also a significant antagonistic effect of exposure X warming, with a lowered N_2O production in the short-term W and NW treatments (Figure 4, Table 2).

When short-term N_2O production rates were normalized for *C. virginica* per wet mass (g) for the short-term exposure period (Table 3), N_2O emissions were significantly affected by nitrogen, warming and their interaction (Table 4).

Dissolved inorganic nitrogen

In the N+W experiment, NH_4^+ and NO_3^- concentrations were generally higher in the N addition and NW treatments for the three bivalve species studied (Figure 5-6), but initial (T_0) concentrations of NH_4^+ and NO_3^- varied between bivalve species despite similar N manipulations in both immediate and short-term exposures.

NH_4^+ concentrations were high in all *M. edulis* treatments and were approximately four times higher compared to the other species studied (Figure 5A). In *M. edulis,* there were unexpectedly high NH_4^+ values in both immediate and short-term exposure even in control treatments, and a decrease of NH_4^+ concentrations in the N treatments during both exposure times (Figure 5A). There was a significant positive effect of nitrogen X warming and exposure on final NH_4^+ concentrations for *M. edulis* (Table 5). The change of NH_4^+ between start and end of incubations was also significantly affected by nitrogen, warming and their interaction (Table 5). In the *M. edulis* aquaria, T_0 and T_5 NH_4^+ concentrations declined as N_2O concentrations increased, in the N addition treatment only (R^2=0.54, p=0.04). NO_3^- concentrations were generally higher in the short-term exposure than the immediate and were higher than expected at the start of the incubation for all treatments (Figure 6A). There was a significant effect of nitrogen, nitrogen X warming and exposure X warming on the final NO_3^- concentrations of *M. edulis* (Table 5). There was no significant treatment or exposure effect on the change of NO_3^- concentration between the start and end of the incubation (Table 5). No relationship was found between N_2O production rates and change in NO_3^- concentrations in the *M. edulis* experiments.

In *M. mercenaria* incubations, initial NH_4^+ (Figure 5B) and NO_3^- concentrations (Figure 6B) were two to three times higher in treatments with N additions, for both immediate and short-term periods (Table 5). Concentrations of NH_4^+ and NO_3^- in all treatments remained constant or increased between time points (Figure 5B, 6B). Final NH_4^+ concentrations were significantly affected by nitrogen, exposure and exposure X nitrogen (Table 5). The change in NH_4^+ concentrations between start and end of incubations were significantly affected by nitrogen, warming, exposure and nitrogen X warming (Table 5). No relationship was found between change in NH_4^+ or NO_3^- and N_2O production. *M. mercenaria* did not show any significant relationships between NH_4^+ concentrations and N_2O concentrations.

C. virginica incubations showed higher NH_4^+ and NO_3^- concentrations at T_0 in the N addition treatments for the immediate and short-term exposures (Figure 5C, 6C). There was also a decrease of NH_4^+ and NO_3^- between T_0 and T_5 for *C. virginica* (Figure 6C). Final concentrations of NH_4^+ + and NO_3^- were significantly affected by all treatments and interactions (Table 5). The change in NH_4^+ was significantly affected by all treatments and interactions except for nitrogen and nitrogen X warming. The change in NO_3^- was significantly affected by nitrogen, exposure, and exposure X nitrogen. For *C. virginica*, T_0 and T_5 NH_4^+ concentrations were significantly increased as N_2O concentrations decreased in the control (R^2=0.43, p=0.03) and in the N addition treatments (R^2=0.47, p=0.02). In contrast, T_0 and T_5 NH_4^+ concentrations increased as N_2O concentrations in the NW treatment increased (R^2=0.61, p<0.01). NO_3^- concentrations also increased as N_2O concentrations increased in the *C. virginica* control (R^2=0.57, p<0.01) and N addition treatments

(R^2=0.44, p=0.03). However, NO_3^- increased while N_2O decreased in the NW treatments (R^2=0.65, p<0.01) driven by differences in nitrate concentrations between immediate and short-term exposures. *C. virginica* NO_3^- concentrations showed significant effects of the warming and combined NW treatments in the short-term exposure (Table 5, Figure 6C).

Dissolved oxygen and pH

Between T_0 and T_5, pH and dissolved oxygen (DO) generally significantly decreased for all organisms and all treatments (Table 6,7). DO reached hypoxic levels (< 3 mg L^{-1}) in *M. edulis* immediate exposure treatments (Table 6). In *M. edulis* N+W experiments, pH was significantly decreased by time points (0 and 5 hours) while experimental treatments did not significantly affect pH. As DO decreased, N_2O increased (R^2=0.51, p<0.01) for *M. edulis*, but no relationship was found between pH and N_2O.

In *M. mercenaria* experiments, pH was significantly affected by time (Table 7), such that pH was lower by the end of the incubation compared to the start (Table 6). pH was also significantly affected by warming and the interaction of warming X time (Table 7).

Time points also affected *C. virginica* pH (Table 7) driven by lower pH at the end of the incubations (Table 6,7). Nitrogen, warming and warming X time also significantly affected pH (Table 7). DO levels were significantly affected by time and warming (Table 6,7). Though levels did significantly drop between start and end of incubations, DO levels did not fall below 3 mg L^{-1} for *C. virginica* in either immediate or short-term incubations (Table 6).

N_2O consumption in seawater (only)

In the control aquaria without invertebrates, N_2O consumption was consistently observed (filtered seawater = -4.2 ± 3.2 nmol N_2O hr^{-1}; filtered seawater with N = -2.7 ± 2.5 nmol N_2O hr^{-1}; raw seawater = -2.7 ± 4.3 nmol N_2O hr^{-1}; raw seawater with N = -20.9 ± 5.9 nmol N_2O hr^{-1}). A significant interaction was found ($F_{3,16}$=4.7, p=0.02), such that the combination of raw seawater with N addition had over fivefold higher rates of N_2O consumption compared to all other combinations of treatments. There was a trend of N addition ($F_{3,16}$=3.2, p=0.09) and water filtration ($F_{3,16}$= 4.3, p=0.06) on N_2O production. There was a significant decrease of pH (t_9=-5.2, p<0.01) and DO levels (t_9= -3.1, p<0.01) by the end of the experimental incubations (Table 6).

Macro epifaunal contributions associated with M. edulis *N_2O production*

The macro-epifauna removal and N-addition experiment with *M. edulis* showed lower average N_2O production rates (Figure 7) than *M. edulis* N+W experiment (Figure 2). There was a significant effect of N treatment on N_2O production ($F_{3,10}$= 6.7, p=0.03). However, there was no significant impact of the presence of macro-epifauna ($F_{3,10}$=2.1, p=0.18) nor the combination of nitrogen X epifauna ($F_{3,10}$=0.03, p=0.87). Although N_2O production rates were qualitatively higher for *M. edulis* with macro-epifauna than those without them, only increased N levels had a significant effect on the rates of N_2O production.

M. edulis +N treatment had the highest average N_2O production rates when normalized per wet mass, at 0.15 nmol N_2O g^{-1} hr^{-1}. Average production rates normalized per wet mass for the remaining treatments were as follows: control (-

epifauna -N) = 0.02 nmol N_2O g^{-1} hr^{-1}, +N treatment= 0.11 nmol N_2O g^{-1} hr^{-1}, and +Epifauna treatment= 0.053 nmol N_2O g^{-1} hr^{-1}.

pH (t_{13}=-7.3, $p<0.01$) and DO levels (t_{13}= -13.6, $p<0.01$) were significantly lower between the start and end of the experiment for the *M. edulis* macro-epifauna removal experiment (Table 6), but DO concentrations did not reach hypoxic levels. N_2O production rates from all treatments showed a weak negative relationship towards pH (R^2=0.37, p=0.02) as well as a weak relationship towards dissolved oxygen (R^2=0.33, p=0.03) of the aquarium water.

Physical condition of bivalves

M. edulis individuals had a 31% mortality rate over the short-term 14-day incubation period. During this period, the control treatments had a total of 3 mortalities and the experimental treatments had 4 mortalities per treatment (out of 12 individuals for each treatment). There was no significant difference in mortality rates per treatment ($F_{3,12}$=0.04, p=0.99). The other species did not experience mortality during this study, nor was there any mortality in the *M. edulis* macro-epifauna experiment.

By the end of the experiment, *M. mercenaria* had the highest condition index (CI) among the three species while *C. virginica* had the lowest (Figure 8). There was a significant interaction between nitrogen and warming on the CI of *M. edulis* ($F_{3,44}$=2.12, p=0.02). *C. virginica* CI was significantly affected by warming (Figure 8, $F_{3,44}$=0.67, $p<0.01$). No significant relationships were found between CI and N_2O production for all taxa between different experimental treatments, but there was a positive relationship for *C. virginica* (R^2= 0.17, $p<0.01$).

There was a weak but significant positive relationship between wet weights of the individuals and N_2O production rates, though was largely driven by large variability in weights and production rates of *M. edulis*.

DISCUSSION

This study revealed that prominent benthic bivalves can enhance water column N_2O, particularly in response to prolonged exposure to fixed N. The increase of N_2O production in the N addition treatments for all three bivalves is reasonable, as more N is available for various metabolic pathways of microbes associated with the bivalves. We found a large range of N_2O among species, from highest N_2O production per individual by *M. edulis* (Figure 2) to N_2O consumption by *C. virginica* (Figure 4). *M. edulis* also produced the highest mass-specific N_2O emissions reaching 7.5 nmol N_2O g^{-1} hr^{-1}, which was unexpected as we hypothesized that the largest species, *M. mercenaria,* would have the largest mass-specific N_2O emissions. It is important to note that the high rates of N_2O production in the N+W *M. edulis* experiments were likely influenced by onset of hypoxia and the organisms' poor physiological status during the five-hour incubation. N_2O responses to N loading and warming are discussed further for each species below.

Nitrogen loading increases N_2O production by bivalves

M. edulis showed the highest production of N_2O among the species studied in the N+W experiment (Figure 2). N_2O from the *M. edulis* N addition treatments were approximately five times higher than *M. mercenaria* and 10-30 times higher than *C. virginica* between exposure times (Figures 2-4). The N_2O production per individual from the -N-W control treatments are an order of magnitude greater than in previous

studies examining *M. edulis* without N or warming manipulations (Steif et al. 2009, Heisterkamp et al. 2013). However, the organisms used in our study were ~60 times heavier (per wet mass) than those used in previous studies, and when compared to mass specific N_2O, our results for the N+W experiment are within the same range as previous studies (Stief et al. 2009; Heisterkamp et al. 2013).

The high N_2O emissions from *M. edulis* may be also due to their high N excretion rates, indicated by the elevated NH_4^+ concentrations of ~50-250 μM NH_4^+-N across all N addition and control treatments (Figure 5A). No other aquaria in this experiment showed such high concentrations of ammonium. This shows that our N pulse was not the dominant source of fixed N for this species. Heisterkamp et al. (2013) directly measured excretion rates of *M. edulis* by examining NH_4^+ accumulation every 0.5 h for 3 h. They found that *M. edulis* N_2O production is sustained through tightly coupled nitrification-denitrification by their own NH_4^+ excretions regardless of water column DIN sources. Though our experiment did not examine specific N_2O-producing processes, we can hypothesize that this NH_4^+ could serve as a substrate for nitrification to occur on their shells or in the water column and may feed coupled nitrification-denitrification. The high declines of NH_4^+ concentrations by the end of the five-hour incubation period in the N and warming treatments (Figure 5A) further indicates that nitrification may have been the prominent N_2O producing process in the immediate exposures. Similarly, for the short-term exposure of 14-days, the high N_2O production associated with N addition treatments was possibly due to nitrification, as there was a decrease in concentrations of NH_4^+ and an increase in concentrations of NO_3^- in the N addition treatments (Figure 5A,

6A). Our findings are consistent with Heisterkamp et al. (2013) who also found higher potential nitrification rates than denitrification rates for *M. edulis* through inhibition-based assays.

Importantly, extrapolation of our results from the N+W *M. edulis* incubations are limited because this species suffered from a drastic decrease in DO and pH concentrations during the five-hour incubation period (Table 6). The significant drop in DO from an average of ~9 mg L^{-1} to 2.5 mg L^{-1} in the immediate exposure means that the high N_2O production rates for *M. edulis* were observed under hypoxic conditions (<3 mg L^{-1}). The high N_2O concentrations from *M. edulis* were negatively related to low DO concentrations suggesting that hypoxic conditions may have been a contributing factor to high N_2O concentrations. It was unlikely the dominant cause of high N_2O emissions since hypoxia and low pH were seen across all treatments whereas only N addition treatments had significantly higher N_2O emissions; thus, the combination of hypoxic conditions and increase N likely stimulated the high N_2O production rates from *M. edulis*. Hypoxia and low pH by the end of the incubation likely induced higher metabolic demands, higher respiration rates and subsequent higher filtration rates to obtain more food for energy from this source population of *M. edulis* (Riisgard and Randløv 1981). There were no DO data available for the short-term duration, however, it is likely to have had a similar drop in DO due to the significant drop in the pH values for *M. edulis* in that incubation (Table 6). It is important to quantify how pH and DO related to N_2O production *in situ,* especially as they are known to covary in areas such as dead zones (Duarte et al. 2013; Altieri and Gedan 2015) and shallow, eutrophic estuaries (Edwards et al. 2004).

M. edulis was also the only species in the N+W experiments to show mortality between immediate and short-term incubations, even though their condition index remained within the range of the other healthy bivalve species in our study (Figure 8). Possibly these indices, though commonly employed in ecological studies (Lee 1996, Lander 2012, Capelle 2014), are not sufficient to detect short term physiological responses that we assayed. The *M. edulis* used in the macro-epifauna removal experiment, which were collected from a different source, did not show such drastic low DO concentrations. Future bivalve associated N_2O production should include quantification of bivalve health and filtration rates, as those may significantly influence how bivalves interact with the overlying water column and resident bacteria.

We expected *M. mercenaria* to be the highest contributor to N_2O production; however, this species showed the second highest bivalve associated N_2O production, whereby N addition treatments significantly increased N_2O production particularly over prolonged exposure to N (Figure 3). Studies have found that filtration rates are significantly lower by *M. mercenaria* when they are assayed in aquaria without sediments compared to those with sediments where they are allowed to burrow (Caughlan and Ansell 1964; Riisgård 1988). Therefore, this species may have not been filter-feeding at high enough rates to store enough denitrifying bacteria in their large guts. This species also did not show evidence of high excretion rates as shown by *M. edulis* (Figure 4), which could have prevented the induction of high rates of coupled nitrification-denitrification processes.

C. virginica showed the lowest overall N_2O production rates, although the N_2O production associated with *C. virginica* in the –N –W control treatments (0.5-2.8 nmol

N_2O ind^{-1} h^{-1}) is the same order of magnitude as other aquatic bivalves (Stief et al. 2009: 200; Heisterkamp et al. 2010; Svenningsen et al. 2012). We hypothesize that consumption of N_2O, which was observed in the immediate control and N addition only treatments, was likely due to complete denitrification. N cycling related to *C. virginica* found that denitrification rates increase in sediments with oyster reefs relative to bare sediments (Piehler and Smyth 2011; Smyth et al. 2013; Kellogg et al. 2014). It is likely that N_2O production will vary when *C. virginica* is assayed with sediments and may in fact increase N_2O when sediment bacteria are allowed to interact with the fixed N.

Another possible reason for N_2O consumption is competition for DIN between N cycling bacteria and phytoplankton in the aquaria (Sundbäck et al. 2000). The phytoplankton density in the unfiltered aquarium water column may have been large enough to outcompete the ammonia oxidizing bacteria and denitrifiers for fixed N (Vieillard and Fulweiler 2014), though this should have been minimized as we performed this study in the dark to minimize the role of phytoplankton. Further, the organisms may have not been excreting at such high rates since there was no NH_4^+ production by the end of the five-hour incubation (Figure 5C). There was minimal consumption of NH_4^+ and NO_3^- (Figure 5C), which also suggests low rates of nitrification and denitrification, respectively.

Warming impact on bivalve N_2O production

Our findings indicate that warming had a slight impact on N_2O emissions associated with these bivalves. *M. edulis* was not significantly affect by warming, however *M. mercenaria* production rates were affected by warming alone and *C.*

virginica only showed significantly warming impact when combined with exposure (Figures 2-4, Table 2). Warming impacts on *M. mercenaria* production rates were likely pulled by the interaction of exposure X warming and exposure X warming X nitrogen (Figure 3, Table 2), since NW treatments showed higher concentrations compared to warming treatment alone. Denitrification rates are typically dependent on both temperature and NO_3^- availability (Stief and Schramm 2010) and thus the combination of higher nitrogen and warming with a longer duration of exposure likely stimulated an increase in N_2O production due to denitrification. It is plausible to predict that highest N_2O emissions associated with this species will occur when NO_3^- availability and temperature coincide in the spring and fall (Jorgensen and Sorensen 1985; Stief and Schramm 2010).

There was a transient increase in N_2O production for *C. virginica* under immediate exposures to warming (Figure 4). In the immediate exposure, the production of N_2O in the NW treatment was possibly due to increased rates of coupled nitrification-denitrification activity since there was higher NH_4^+ and NO_3^- decrease compared to the non-warming treatments (Figure 5C, 6C). There was less NH_4^+ in the short-term NW treatment compared to the immediate exposure NW treatment (Figure 5C), which may not have given the ammonia oxidizing bacteria enough substrate to metabolize even under warming conditions.

No known studies have thus far examined warming impact on N_2O production from bivalve species, though studies have examined warming impacts on bivalve health and growth (Lannig et al. 2006; Hiebenthal et al. 2012; Matoo et al. 2013; Mackenzie et al. 2014). Optimal temperatures for denitrifiers in marine sediments are

21-36ºC (Canion et al. 2014), while our water temperatures were on the low end of that range at 22ºC. In addition, warming may have more substantial effects over longer time periods.

Macro-epifauna contribution to M. edulis *N_2O production*

Unlike our hypothesis, results of the macro-epifauna removal and N addition experiments (Figure 7) suggest that macro-epifauna organisms do not significantly contribute to N_2O production of *M. edulis* in Narragansett Bay. Shell microbial biofilms may have contributed to N_2O production, explaining the discrepancy between our results and past research that has found shell biota to be significant contributors (Heisterkamp et al. 2010; Svenningsen et al. 2012; Heisterkamp et al. 2013).

M. edulis individuals from the epifaunal removal experiment (Figure 7) also had lower N_2O production when compared to the N+W experiment (Figure 2), and the former were more consistent with previous production rates of *M. edulis* (Stief et al. 2010; Heisterkamp et al. 2010, 2013). We hypothesize that the difference between these experiments is due to the filtered vs. unfiltered water used, respectively. N_2O production in the epifauna (with no N addition, Figure 7) treatment was ~3 times lower than immediate exposure control results for *M. edulis* in the NW experiment (Figure 2). The N_2O production from the epifauna + nitrogen addition treatment was also ~3 times lower than *M. edulis* N addition treatment in the N+W experiment. Filtration of the water column likely removed N_2O producing bacteria that *M. edulis* would normally ingest from the water column, therefore lowering the N_2O production rate associated with *M. edulis*. Stief (2013) suggests microbes ingested by bivalves may not be digested and survive in the gut, remaining metabolically active to facilitate

production of N_2O. *M. edulis* is an efficient filter feeder that ingests large amounts of bacteria (McHenery and Birkbeck 1985); therefore, the rates described associated with *M. edulis* in the epifauna removal experiment (Figure 7) are likely produced from N_2O producing microbes already present within the gut of the organism before the start of this experiment as well as potential microbial biofilms on their shells.

CONCLUSION

Our study illustrates that N loading increases N_2O emissions from bivalve shellfish. We show little evidence that warming or macro-epifauna significantly contribute to N_2O production in this short term lab study, however future longer duration experiments are needed to better simulate the natural environment. Potential N_2O emissions from bivalves differed between species and were not biomass dependent as has been shown for other invertebrate taxa. *M. edulis* produced the highest rates of N_2O rather than *Mercenaria mercenaria,* which we believe to be a combination of high NH_4^+ production and the induction of hypoxic conditions due to potentially poor health of one source population. Notably, N_2O emissions increased via exposure to N loading and will possibly further increase in natural systems where they can interact with marine sediments and increase benthic N cycling. Since marine bivalves are found in high abundances in coastal systems where the combination of eutrophic, warming and low DO often coincide, their potential contribution to N_2O emissions from benthic systems may be higher than previously understood and warrants further investigation.

TABLES AND FIGURES

Table 1. Water quality parameters of field sites at time of invertebrate collection for Nitrogen + Warming manipulation experiments.

Site	Depth	Ammonia (µM)	Nitrate (µM)	pH	DO (mg L^{-1})	Temperature (ºC)	Salinity (ppt)
Conimicut Point	Surface	31.54	1.78	8.02	7.52	22.73	28.42
	Bottom	22.72	2.27	7.69	4.15	20.54	32.67
Narragansett	Surface	25.00	0.30	7.9	7.19	21.87	30.46
	Bottom	24.36	1.58	7.8	5.93	21.63	29.98
North Kingstown	Surface	26.46	0.59	8.1	7.19	15.17	30.78
	Bottom	22.63	1.28	7.9	6.32	12.34	31.01

Table 2. Summary of mixed model analysis for impacts of Nitrogen + Warming manipulation experiments on N_2O production rates (nmol ind^{-1} hr^{-1}) for the 3 bivalves. *statistical significance at $\alpha = 0.05$

	M. edulis		*M. mercenaria*		*C. virginica*	
Parameter	***F*-ratio**	**p-value**	***F*-ratio**	**p-value**	***F*-ratio**	**p-value**
N	$F_{1,20}$=30.6	<0.0001*	$F_{1,22}$=113.6	<0.0001*	$F_{1,24}$=6.6	0.02*
W	$F_{1,20}$=0.1	0.83	$F_{1,22}$=7.4	0.01*	$F_{1,24}$=3.6	0.07
N*W	$F_{1,20}$=1.7	0.20	$F_{1,22}$=3.4	0.08	$F_{1,24}$=2.3	0.14
Exposure	$F_{1,20}$=62.2	<0.0001*	$F_{1,22}$=8.8	0.01*	$F_{1,24}$=3.6	0.07
Exposure*N	$F_{1,20}$=15.6	<0.001*	$F_{1,22}$=13.9	<0.01*	$F_{1,24}$=0.5	0.49
Exposure*W	$F_{1,20}$=0.2	0.68	$F_{1,22}$=13.0	<0.01*	$F_{1,24}$=18.2	<0.001*
Exposure*N*W	$F_{1,20}$=1.2	0.28	$F_{1,22}$=19.0	<0.001*	$F_{1,24}$=1.6	0.22

Table 3. Potential N_2O emission rates per wet mass (g) of each species (mean ± standard error) for short-term Nitrogen + Warming experimental treatments.

	N_2O emission (nmol g^{-1} hr^{-1})		
Treatment	***M. edulis*** (14-days)	***M. mercenaria*** (28-days)	***C. virginica*** (28-days)
Control	0.74 ± 0.27	0.004 ± 0.01	-0.04 ± 0.06
Nutrient	5.22 ± 1.21	0.34 ± 0.12	0.29 ± 0.06
Warming	1.65 ± 0.81	0.01 ± 0.02	-0.01 ± 0.04
Nutrient + Warming	2.57 ± 0.53	0.10 ± 0.04	0.01 ± 0.05

Table 4. Summary of two-factor ANOVA analysis for impacts of Nitrogen + Warming manipulation experiments on short-term N_2O biomass normalized production rates (nmol g^{-1} hr^{-1}) for the 3 bivalves. *statistical significance at $\alpha = 0.05$

	M. edulis		*M. mercenaria*		*C. virginica*	
Parameter	**F-ratio**	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
N	$F_{3,9}$= 6.9	0.03*	$F_{3,12}$=11.6	0.005*	$F_{3,12}$= 10.0	0.01*
W	$F_{3,9}$=0.3	0.61	$F_{3,12}$=3.5	0.08	$F_{3,12}$=5.0	0.05*
N*W	$F_{3,9}$=6.1	0.04*	$F_{3,12}$=3.7	0.08	$F_{3,12}$=7.8	0.02*

Table 5. Results of mixed model analysis for comparison of final ammonium and nitrate concentrations between treatments (after 5 hour incubation) and change between the beginning and end of each 5 hour incubation in the Nitrogen + Temperature manipulation experiments. *statistical significance at $\alpha = 0.05$

	M. edulis		*M. mercenaria*		*C. virginica*	
Parameter	**F-ratio**	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
Ammonium at end of incubation (5 hour)						
N	$F_{1,8}$=1.64	0.21	$F_{1,8}$=73.53	<0.001*	$F_{1,14}$=648.0	<0.0001*
W	$F_{1,8}$=0.36	0.55	$F_{1,8}$=1.21	0.30	$F_{1,14}$=7.94	0.01*
N*W	$F_{1,8}$=45.6	<0.0001*	$F_{1,8}$=0.48	0.50	$F_{1,14}$=38.0	<0.0001*
Exposure	$F_{1,8}$=91.46	<0.0001*	$F_{1,8}$=5.85	0.05*	$F_{1,14}$=375.3	<0.0001*
Exposure*N	$F_{1,8}$=.0.15	0.7	$F_{1,8}$=8.38	0.02*	$F_{1,14}$=145.7	<0.0001*
Exposure*W	$F_{1,8}$=12.24	12.24	$F_{1,8}$=2.63	0.14	$F_{1,14}$=21.43	<0.0004*
Exposure*N*W	$F_{1,8}$=12.26	12.26	$F_{1,8}$=1.21	0.31	$F_{1,14}$=14.86	<0.002*
Nitrate at end of incubation (5 hour)						
N	$F_{1,8}$=44.89	<0.0001*	$F_{1,8}$=64.0	<0.0001*	$F_{1,14}$=2084.6	<0.0001*
W	$F_{1,8}$=4.6	0.05*	$F_{1,8}$=0.5	0.51	$F_{1,14}$=71.0	<0.0001*
N*W	$F_{1,8}$=4.3	0.05*	$F_{1,8}$=0.07	0.07	$F_{1,14}$=151.4	<0.0001*
Exposure	$F_{1,8}$=78.64	<0.0001*	$F_{1,8}$=0.5	<0.0001*	$F_{1,14}$=1109.1	<0.0001*
Exposure*N	$F_{1,8}$=2.9	0.10	$F_{1,8}$=6.3	0.04*	$F_{1,14}$=501.03	<0.0001*
Exposure*W	$F_{1,8}$=7.1	0.01*	$F_{1,8}$=1.0	0.34	$F_{1,14}$=60.52	<0.0001*
Exposure*N*W	$F_{1,8}$=3.9	0.06	$F_{1,8}$=5.3	0.05*	$F_{1,14}$=142.84	<0.0001*
Ammonium change (T_5-T_0)						
N	$F_{1,8}$= 5.31	0.05*	$F_{1,8}$= 6.42	0.04*	$F_{1,14}$=0.86	0.37
W	$F_{1,8}$=5.17	0.05*	$F_{1,8}$=12.28	0.01*	$F_{1,14}$=5.28	0.04*
N*W	$F_{1,8}$=6.82	0.03*	$F_{1,8}$=7.25	0.03*	$F_{1,14}$=0.64	0.44
Exposure	$F_{1,8}$=12.80	0.007*	$F_{1,8}$=2.34	0.16	$F_{1,14}$=16.20	0.001*
Exposure*N	$F_{1,8}$=2.72	0.14	$F_{1,8}$=14.24	0.01*	$F_{1,14}$=4.61	0.05*

Exposure*W	$F_{1,8}$=1.15	0.31	$F_{1,8}$=6.39	0.04*	$F_{1,14}$=39.68	<0.0001*
Exposure*N*W	$F_{1,8}$=1.80	0.22	$F_{1,8}$=2.97	0.12	$F_{1,14}$=6.57	0.02*
Nitrate change (T_5-T_0)						
N	$F_{1,8}$=3.75	0.09	$F_{1,8}$=11.92	0.009*	$F_{1,14}$= 22.68	<0.001*
W	$F_{1,8}$=1.69	0.23	$F_{1,8}$=0.65	0.44	$F_{1,14}$=0.46	0.51
N*W	$F_{1,8}$=0.35	0.57	$F_{1,8}$=2.88	0.13	$F_{1,14}$=2.01	0.18
Exposure	$F_{1,8}$=0.02	0.89	$F_{1,8}$=31.75	<0.001*	$F_{1,14}$= 26.1	<0.001*
Exposure*N	$F_{1,8}$=4.39	0.07	$F_{1,8}$=0.36	0.36	$F_{1,14}$=14.53	0.002*
Exposure*W	$F_{1,8}$=1.54	0.25	$F_{1,8}$=0.67	0.67	$F_{1,14}$=1.71	0.21
Exposure*N*W	$F_{1,8}$=0.15	0.71	$F_{1,8}$=2.52	0.15	$F_{1,14}$=2.58	0.13

Table 6. Average pH and dissolved oxygen (DO) values at the start (T_0) and end (T_5) of each experimental incubation period.

Experiment	Species	Exposure	pH		DO (mg L^{-1})	
			T_0	T_5	T_0	T_5
N+W	*M. edulis*	Immediate	8.0 ± 0.1	7.3 ± 0.1	9.1 ± 0.04	2.7 ± 0.19
		Short-term	7.8 ± 0.1	7.3 ± 0.1	NA	NA
N+W	*M. mercenaria*	Immediate	8.1 ± 0.1	7.7 ± 0.1	NA	NA
		Short-term	7.9 ± 0.1	7.8 ± 0.1	NA	NA
N+W	*C. virginica*	Immediate	8.0 ± 0.1	7.4 ± 0.1	9.2 ± 0.10	7.3 ± 0.15
		Short-term	8.0 ± 0.1	8.1 ± 0.1	9.2 ± 0.10	8.1 ± 0.11
Water only	None		8.2 ± 0.1	8.1 ± 0.1	9.7 ± 0.04	9.6 ± 0.01
Epifauna+N	*M. edulis*		7.9 ± 0.1	7.6 ± 0.1	9.3 ± 0.04	7.6 ± 0.13

Table 7. Mixed model analysis for pH and dissolved oxygen (DO) in the N+W experiment. N=Nitrogen, W=Warming, T= Time point (0, 5 hours). *statistical significance at α = 0.05

	M. edulis		*M. mercenaria*		*C. virginica*	
Parameter	**F-ratio**	**p-value**	**F-ratio**	**p-value**	**F-ratio**	**p-value**
pH						
N	$F_{1,48}$=3.7	0.06	$F_{1,56}$=0.47	0.49	$F_{1,56}$=5.3	0.03*
W	$F_{1,48}$=0.002	0.97	$F_{1,56}$=13.8	<0.001*	$F_{1,56}$=5.4	0.02*
N*W	$F_{1,48}$=0.02	0.89	$F_{1,56}$=0.06	0.81	$F_{1,56}$=2.9	0.09
T	$F_{1,48}$=199.0	<0.0001*	$F_{1,56}$=80.1	<0.0001*	$F_{1,56}$=50.8	<0.0001*
T*N	$F_{1,48}$=0.002	0.97	$F_{1,56}$=1.24	0.27	$F_{1,56}$=3.5	0.07
T*W	$F_{1,48}$=1.9	0.18	$F_{1,56}$=9.7	0.003*	$F_{1,56}$=5.2	0.03*

T*N*W	$F_{1,48}$=0.8	0.39	$F_{1,56}$=0.001	0.93	$F_{1,56}$=3.5	0.07
Dissolved Oxygen						
N	$F_{1,48}$=0.2	0.7	NA	NA	$F_{1,56}$=0.06	0.80
W	$F_{1,48}$=0.1	0.79	NA	NA	$F_{1,56}$=75.0	<0.0001*
N*W	$F_{1,48}$=0.2	0.64	NA	NA	$F_{1,56}$=0.40	0.53
Time	$F_{1,48}$=969.2	<0.0001*	NA	NA	$F_{1,56}$=271.9	<0.0001*
T*N	$F_{1,48}$=0.2	0.65	NA	NA	$F_{1,56}$=0.23	0.63
T*W	$F_{1,48}$=1.6	0.22	NA	NA	$F_{1,56}$=0.03	0.86
T*N*W	$F_{1,48}$=0.1	0.72	NA	NA	$F_{1,56}$=0.42	0.52

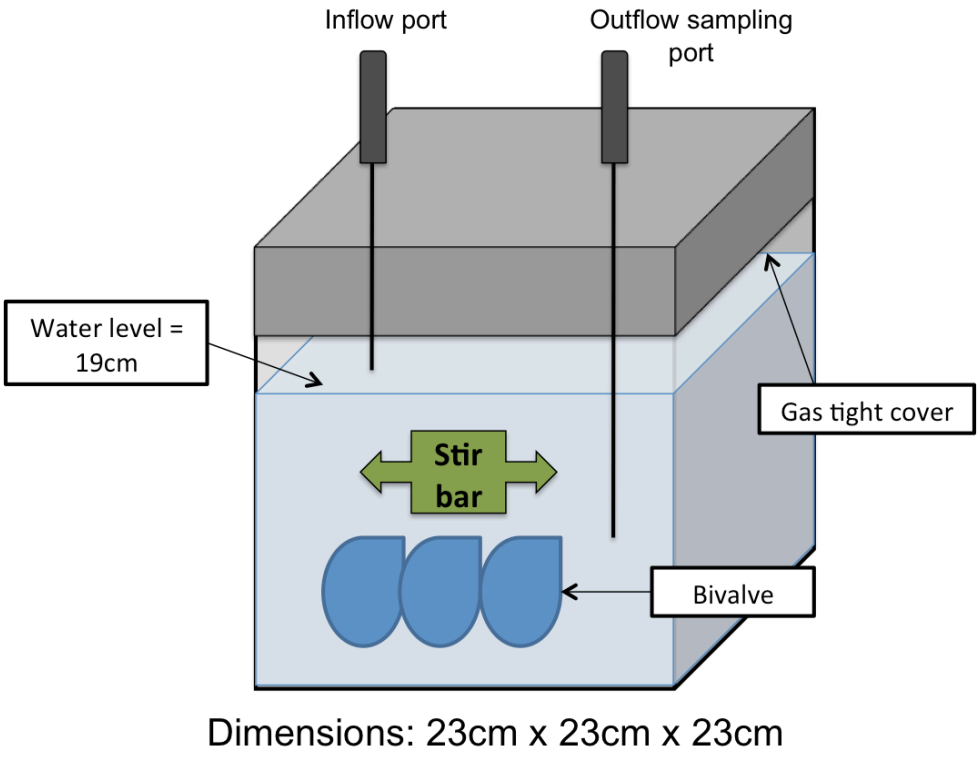

Figure 1. Invertebrate mesocosm diagram.

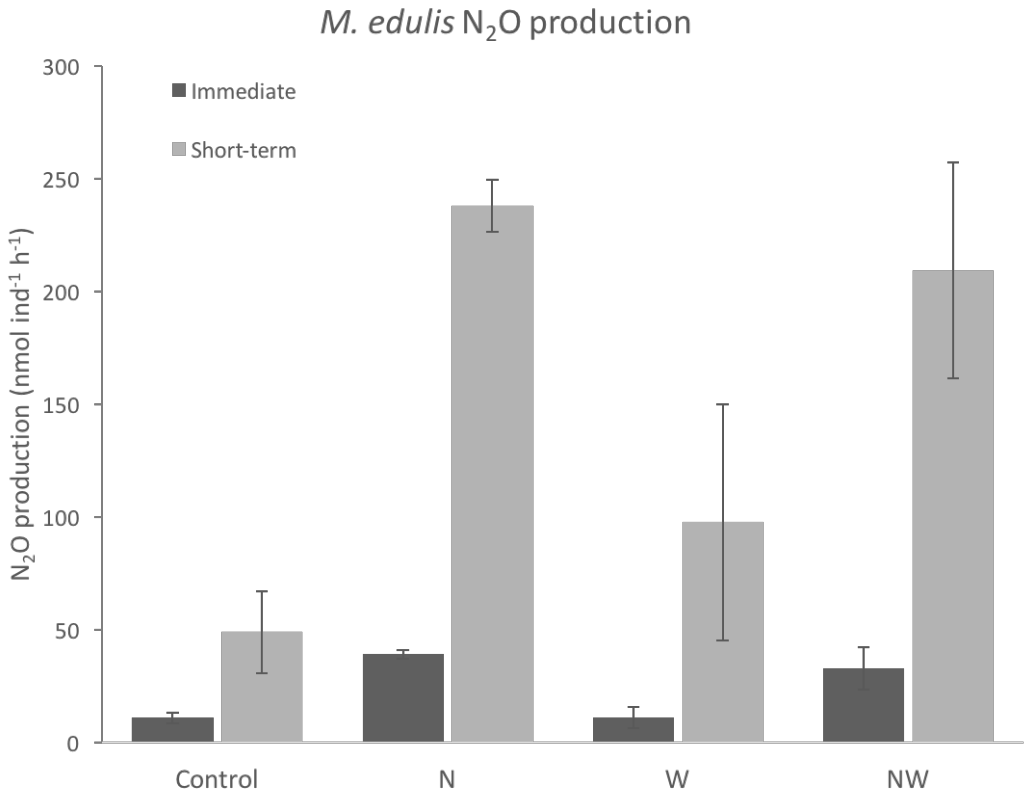

Figure 2. *Mytilus edulis* N_2O production for immediate exposure (1-day) and short-term exposure (28-days). N= Nitrogen addition, W= Warming, NW= Nitrogen + Warming. Error bars show standard error.

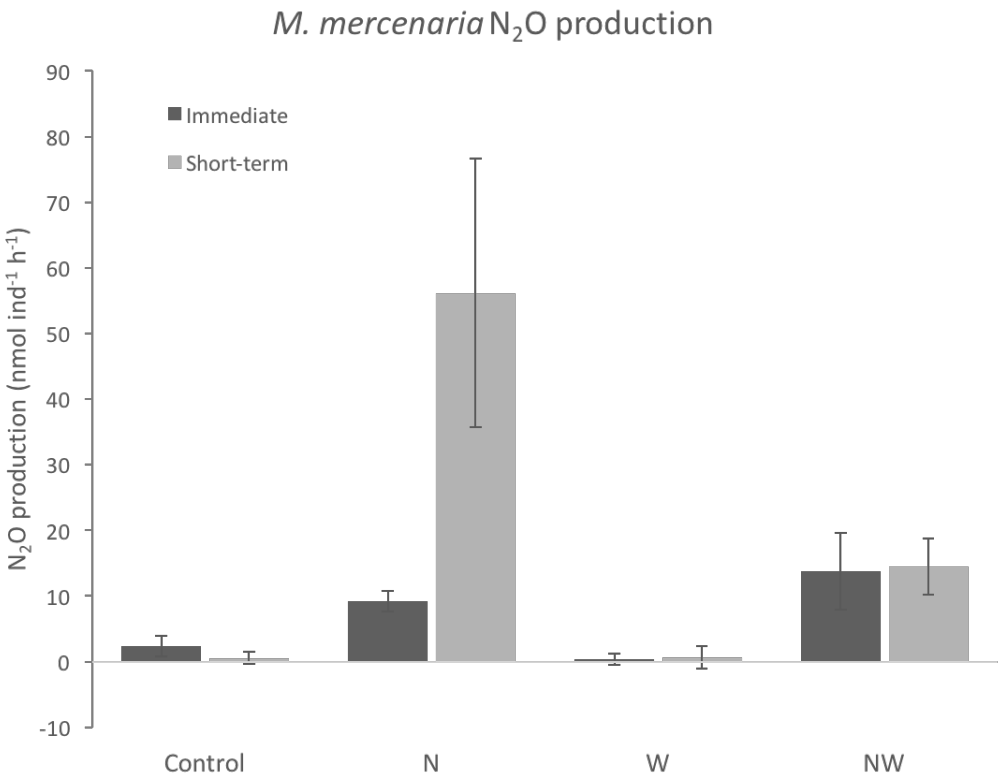

Figure 3. *Mercenaria mercenaria* N_2O production for immediate exposure (1-day) and short-term exposure (28-days). N= Nitrogen addition, W= Warming, NW= Nitrogen + Warming. Error bars show standard error.

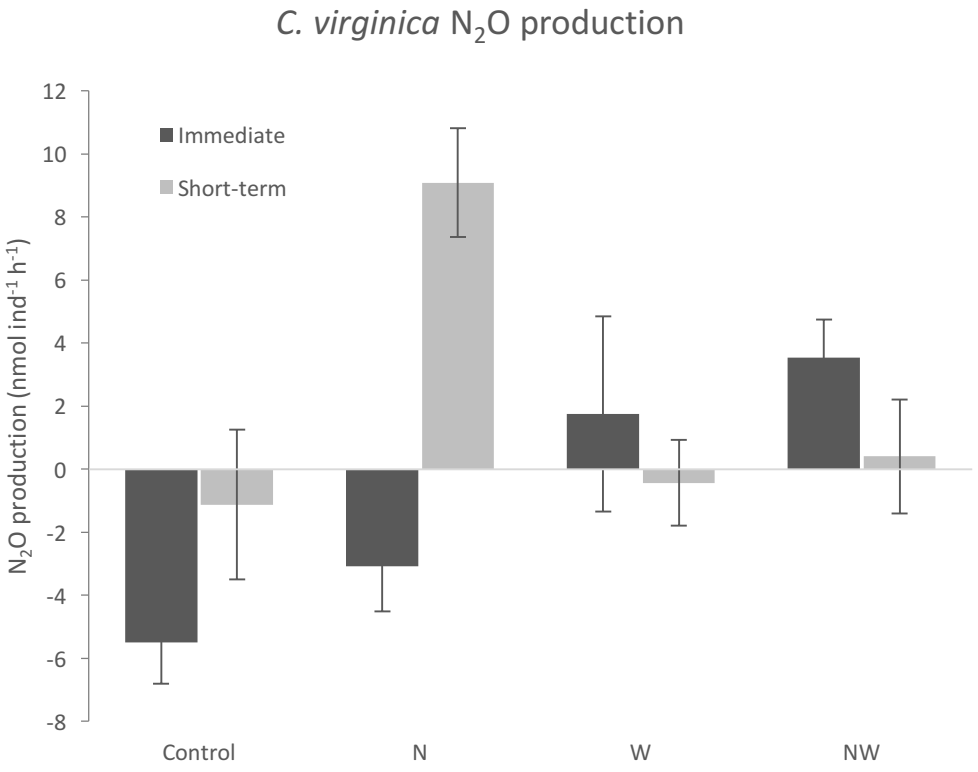

Figure 4. *Crassostrea virginica* N_2O production for immediate exposure (1-day) and short-term exposure (28-days). N= Nitrogen addition, W= Warming, NW= Nitrogen + Warming. Error bars show standard error.

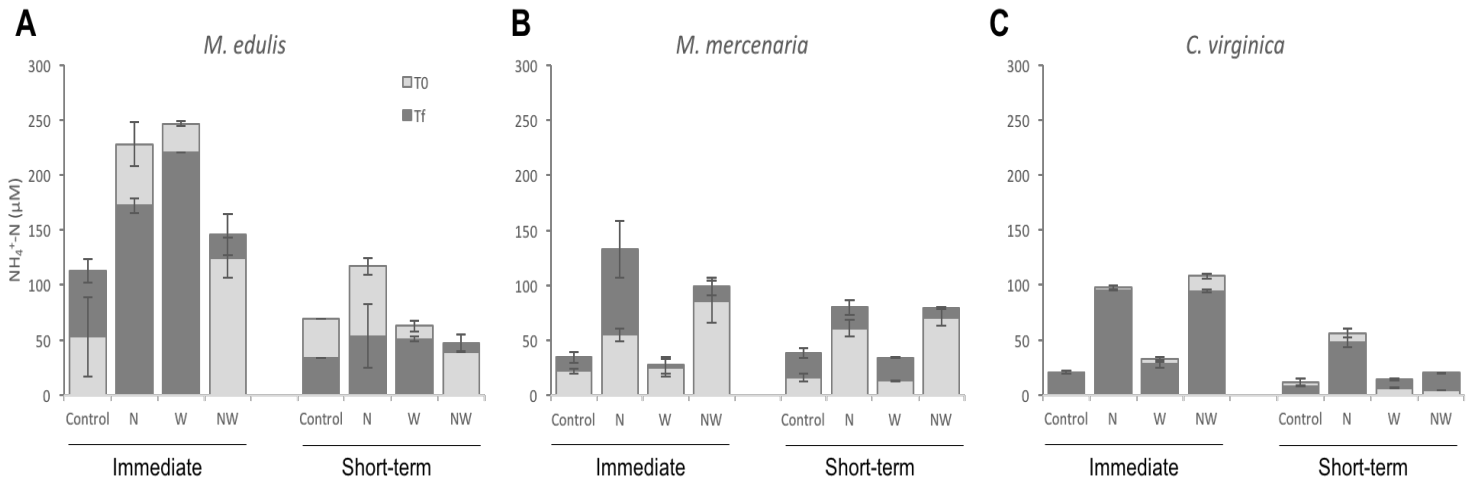

Figure 5. Ammonium concentrations for Nitrogen + Warming experiment at start (T_0) and end (T_f) of each incubation for the three bivalve species: (A) *Mytilus edulis;* (B) *Mercenaria mercenaria;* (C) *Crassostrea virginica* treatments. Standard error bars shown. N= Nitrogen

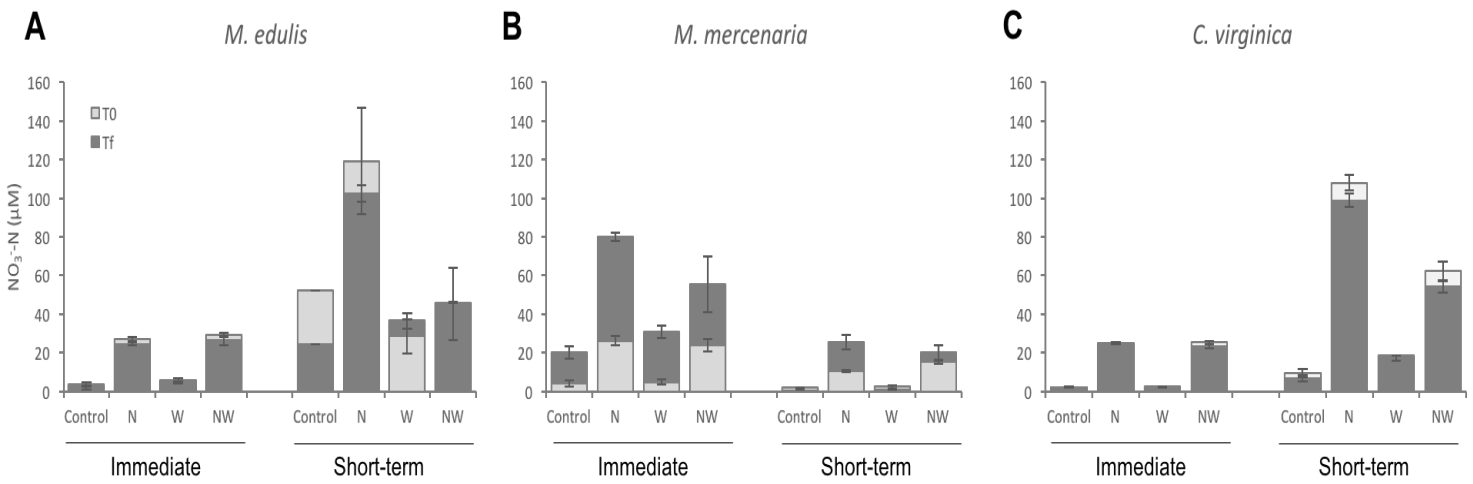

Figure 6. Nitrate concentrations for Nitrogen x Warming experiment at start (T_0) and end (T_f) of each incubation for the three bivalve species: (A) *Mytilus edulis; (B) Mercenaria mercenaria; (C) Crassostrea virginica.* N= Nitrogen addition, W= Warming, NW=Nitrogen + warming. Light bar= initial (T_0) and dark bar= final (T_5).

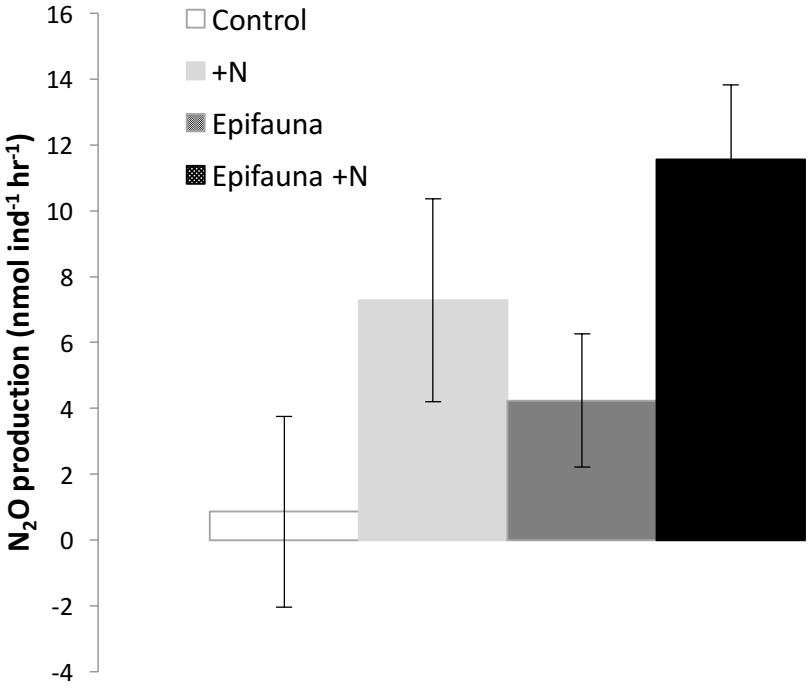

Figure 7. Average N_2O production rates from *M. edulis* exposed to: Control= no macro-epifauna, no N addition; +N= macro-epifauna removed, N addition; Epifauna= macro-epifauna present, no N addition; Epifauna+ N= macro-epifauna present, N addition. Error bars show standard error.

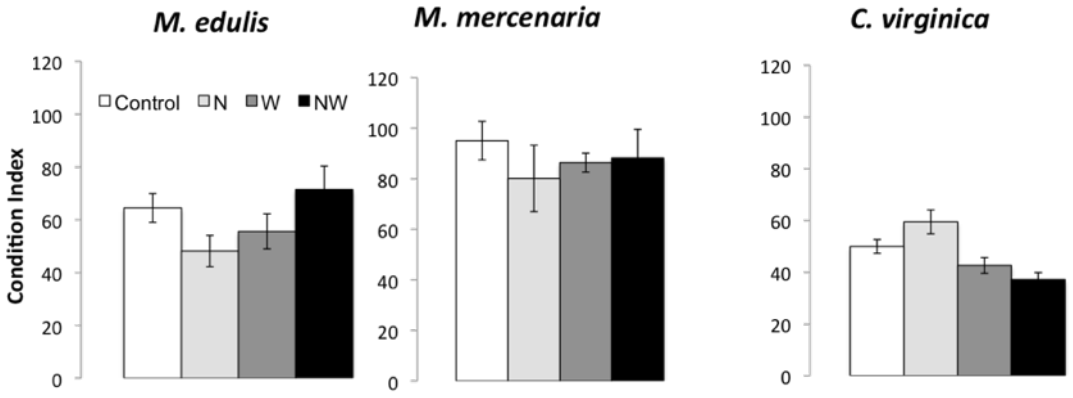

Figure 8. Condition index ratio per species for Nitrogen + Warming experiment. Error bars show standard error. N= Nitrogen, W= Warming, NW= Nitrogen + Warming.

APPENDICES

Appendix I

Rhizophora mangle sediments are sources of potent greenhouse gases in Jobos Bay, Puerto Rico

Carbon dioxide (CO_2), nitrous oxide (N_2O) and methane (CH_4) are potent greenhouse gases (GHGs) that are readily increasing in the atmosphere due to human activities and are significant drivers of climate change (Forster et al. 2007). Mangrove wetlands are important ecosystems for potentially mitigating climate change as they have significant carbon stocks (Donato et al. 2011). However, with large inputs of human-derived pollutants such as nitrogen (N), mangroves may shift from being sinks of GHGs to sources (Corredor et al. 1999; Muñoz-Hincapié et al. 2002; Chen et al. 2012). The purpose of this experiment was to compare GHG emissions from *Rhizophora* dominant mangrove sediments in Jobos Bay, Puerto Rico at a pristine site and an anthropogenically influenced site. We hypothesized higher GHGs emissions from mangrove sediments in the anthropogenically influenced site compared to the pristine. We also wanted to determine if pulses of nitrogen (N) would stimulate metabolism by N-cycling bacteria and produce significant amounts of N_2O.

METHODS

Greenhouse gas emissions were measured in the red mangrove zone in Jobos Bay, Puerto Rico at a pristine site (Cayes) and an anthropogenically influenced site (Mar Negro). Jobos Bay is on the south coast of the island and dominated by agriculture. Mar Negro is directly influenced by human pollutants as it is adjacent to a residential area that commonly disposes of their untreated human waste directly on the

mangrove watershed (Bowen and Valiela 2008). The pristine site is one of fifteen small mangrove fringe islands that have no direct connection to the mainland and therefore little anthropogenic influence. These measurements were collected during the wet season, though there was a drought occurring in this region of Puerto Rico during this time. Each site had 6 replicates that were ~1m apart from each other. Fluxes were measured using a cavity ring-down spectrophotometer (Picarro G2508) during low tide using 100 foot tubing that was used to connect the analyzer to a static flux chamber. The chamber (volume approximately 10 liters) was equipped with an inflow port, outflow port, fan to homogenize the air and pigtail to equalize pressure. The chamber was placed on the mangrove sediment on preinstalled (2 days) aluminum collars for 8 minutes. GHG fluxes were calculated using the Ideal Gas Law (PV = nRT) using field-measured air temperatures (HOBO, Inc) and atmospheric pressure. If there was no change in gas concentration over time during the measurement period, fluxes were reported as negligible.

Salinity, pH, soil moisture, and redox potential were measured to determine correlations between edaphic parameters and gas fluxes. Soil salinity was measured from the top 3cm by pressing the soil against paper filters within a plastic syringe (15mL). The extracted water was then measured for salinity on a handheld portable refractometer. To measure pH, a soil slurry was made by collecting approximately 10 mL of surface soil using a cut off syringe and adding it to a 50mL falcon tube with 15mL of deionized water. Soil pH was then measured using a pH meter (Thermo Scientific Orion Star A326 pH/Dissolved Oxygen Portable Multiparameter Meter) after the slurry was shaken for approximately 10 seconds. Soil moisture content was

measured using a volumetric water content sensor (Decagon Devices, Pullman, WA) inserted 5 cm into the soil and soil oxidation reduction (redox) potential was measured using a pH/ORP meter (Metler Toledo, Greifensee, Switzerland). 10 cm deep porewater (5 mL) was obtained using rhizons (Rhizosphere), filtered with a 0.45 μm filter and frozen in order to measure dissolved inorganic nitrate (DIN).

In order to examine if nutrient pulses stimulate N_2O fluxes, we also performed a N enrichment experiment in Mar Negro. N treatments received 500 mL of 300 μM potassium nitrate made with unfiltered site water which had salinities of 31ppt prior to flux measurement. Control plots received 500 mL of site water only. Each treatment had three replicates. N manipulations and seawater controls were applied by slowing pouring the amount over the designated plot area. Fluxes were measured within one minute of experimental manipulations, 1.5 hours later, and 72 hours (3 days) later using methods as described above. Due to variability in raw data, N_2O fluxes were calculated using twenty second blocked averages. Edaphic parameters and porewater were measured as described above immediately after gas flux measurements.

STATISTICAL ANALYSIS

A paired t-test was used to determine statistical significant differences for each greenhouse gas and edaphic parameters between sites. A two-factor ANOVA was used to determine if there was a statistical significant influence on N_2O fluxes by N pulse and time (1 factor=treatment, 2 factor=time).

Unequal variance and normality were tested using the Bartlett test and Shapiro-Wilk test, respectively, in order to ensure assumptions were met for each statistic. Correlations were used to test relationships between edaphic parameters and

greenhouse gas fluxes.

All statistical analyses were performed with JMP 10.0 software and significant levels of α=0.05 were used.

RESULTS AND DISCUSSION

Our study found that CH_4 and CO_2 fluxes from mangrove sediments were positive at both sites, while N_2O was negligible. CO_2 fluxes were significantly higher ($t_{1,10}$=5.07, p=0.05) in the anthropogenically influenced site compared to the pristine (Figure 1). A similar pattern was found for CH_4 ($t_{1,10}$=18.42, p<0.01) such that CH_4 fluxes from the anthropogenic site was ~11x higher than the pristine.

Edaphic results are listed in Table 1. There were no significant relationships between edaphic parameters and GHG fluxes, except for a weak positive relationship between soil moisture and CO_2 fluxes in the pristine site (R^2=0.67, p=0.05). Salinity was significantly higher in the pristine site compared to the anthropogenic site ($t_{1,10}$=2.18, p=0.05), likely due to its closer proximity to the open ocean. All other edaphic parameters were similar between sites. Porewater ammonium concentrations were higher in the anthropogenically influenced site and were positively related to CH_4 fluxes (R^2=0.50, p=0.03), suggesting anthropogenic nitrogen inputs may potentially enhance microbial metabolism and enhance GHG emissions from these mangroves.

Our study found no effects of N additions on CH_4 or CO_2 at 0, 1.5 hours or 72 hours. N pulses did significantly increase N_2O emissions compared to controls at 1.5 and 72 hours ($F_{3,7}$=4.6, p=0.01), though the time or the combination of time and treatment did not have a significant affect (Figure 2). N_2O flux was negligible at 0

hours (not shown). Increase in N_2O suggests that microbial metabolism was enhanced with the N pulse. Since redox levels were negative (Table 1), denitrification was the likely source of N_2O emissions as denitrification occurs in anoxic conditions (Zumft 1997).

These fluxes are toward the lower end of the GHG flux range compared to other anthropogenically impacted mangroves ecosystems (Chauhan et al. 2008; Chen et al. 2012; Call et al. 2015). This may be due to differences in hydrology of the sites (diurnal tide cycle) or C:N ratio of the sediments. Altering oxic to anoxic conditions (ie: semi-diurnal tides) can result in higher denitrification rates from sediments than those under relatively more continuous anoxic conditions (diurnal tides). Higher sediment C:N typically yield higher respiration rates (Rivera-Monroy and Twilley 1996), thus further analysis is needed to determine the potential influence of C:N on GHG fluxes. Further analysis is also needed during different seasonal and temporal scales to quantify the extent of their potential affect on carbon sequestration rates.

Appendix II

Greenhouse gas fluxes associated with salt marsh dieback

Salt marsh habitats hold many ecosystem services such as storm protection, nursery habitats and carbon sequestration (Chmura et al. 2003). Unfortunately, these vital ecosystems are experiencing significant dieback events worldwide due to direct and indirect anthropogenic disturbances, particularly in regards to climate change (Alber et al. 2008). Dieback in New England salt marshes, for example, have been

attributed to increases in herbivory by crabs whose populations have increased due to human-induced decrease in predatory pressure (Bertness et al. 2009; Holdredge et al. 2009; Altieri et al. 2012). Another major concern is rising sea level, which causes prolonged inundation of salt marsh grasses, eventually leading to plant loss and shifts in plant community structure (Craft et al. 2009). Dieback events may significantly alter the ecosystem services of a saltmarsh, including greatly reducing ability to sequester carbon and become sources rather than sinks of greenhouse gases (GHGs).

The purpose of our experiment was to examine the combined effects of crab behavior and sea level rise on the greenhouse gas fluxes of the dominant high marsh plant, *Spartina patens* in a mesocosm study. We focused on the herbivorous crab *Sesarma* and also on the burrowing decapod, *Uca puglator*, which may also have a significant impact on the salt marsh soils though have been largely understudied. We hypothesized that *Sesarma* would significantly increase GHG emissions (carbon dioxide and methane) due to its herbivory activity while *Uca* would increase the carbon sequestration rates as it has been positively related to salt marsh plant health. We also examined the GHG fluxes of a New England salt marsh that is currently undergoing a dieback event compared to its intact area *in situ*. We hypothesized that the salt marsh dieback zone would have significantly higher GHG emissions compared to the intact zone (control), due to the reduction of photosynthesis by plants and subsequent increase in microbial respiration in the soils.

METHODS

Our mesocosm experiment was conducted at the Environmental Protection Agency greenhouse facility in Narragansett, RI using six flow-through mesocosm

tanks (900L) under a semi-diurnal tidal regime (Oczkowski et al. 2015). Male *Sesarma* were collected at Colt State Park in Bristol, RI and male *Uca pugilator* were collected at Mary's Creek in Warwick, RI. Each of the tanks had six pots (34.5 cm diameter, 24.5 cm height, 0.09 m^2 area) with soil and three small plugs of the graminoid *Spartina patens* planted in the center (Environmental Concern Inc.). Each tank had two inundation treatments (high, low), combined with three crab treatments (control, *Sesarma, Uca).* Two crabs were placed in each pot for each of the inundation levels. During the simulated high tide, the low inundation treatments were saturated from underneath while the soil surface of the high inundation level was fully submerged. The experiment was conducted from 30 June 2014 to 18 August 2014.

GHG measurements were taken using a cavity ringdown spectrometer (Picarro G2508) in three randomly selected tanks on two dates (2 and 31 July 2014), which were approximately in the beginning and end of the fall season. Nylon tubing (50 feet) was used to connect the analyzer to a static flux chamber for GHG measurements. GHG measurements and edaphic parameters (pH, redox, soil moisture, salinity) were measured as stated in Appendix I. The only exception is that a larger chamber (~13 L) was used for this study.

GHG measurements were made in a dieback and an intact salt marsh zone in Passeonkquis Cove, Rhode Island (41°44'45.0"N, 71°23'25.5"W) on during early (October) and late (December) fall. The dieback region was characterized by a visual reduction of *Spartina alterniflora* aboveground biomass and was largely comprised of organic soils. The intact zone was comprised of visually healthy and continuous low marsh *Spartina alterniflora*. Each zone had four replicates at least 1m apart. GHG

measurements were taken as stated above for the mesocosm study, with the exception that the intact zone measures were taken on preinstalled collars since plants and roots would normally obstruct a gas tight seal. Edaphic parameters were measured as stated above with the exception that soil temperature was also measured for this experiment by inserting a handheld soil thermometer 5cm into the soil.

STATISTICAL ANALYSIS

A two-factor ANOVA was used to determine statistical significant differences in greenhouse gases and in edaphic parameters between mesocosm experimental treatments (1 factor= crab, 2 factor=inundation). A two-factor ANOVA was also used to determine statistical significance in *in situ* salt marsh study (1 factor= treatment, 2 factor=time of data collection). Unequal variance and normality were tested using the Bartlett test and Shapiro-Wilk test, respectively, in order to ensure assumptions were met for the two-factor ANOVA.

Correlations were used to test relationships between edaphic parameters and greenhouse gas fluxes.

All statistical analyses were performed with JMP 10.0 software and significant levels of α=0.05 were used.

RESULTS

Positive carbon dioxide (CO_2) fluxes were significantly affected by inundation on 2 July 2014, but were not affected by crab presence or the combination (Figure 3A, Table 2). The low inundation treatments had higher CO_2 emissions compared to the control (Figure 3A). Towards the end of the experiment on 31 July 2014, inundation

did not individually impact CO_2 fluxes, however crab and the combination did significantly affect CO_2 (Figure 3B, Table 2). The crab control (no crabs) treatment under low inundation showed similar CO_2 uptake compared to *Uca* low inundation treatments, while *Sesarma* treatments showed production of CO_2. Our hypothesis was partly confirmed such that CO_2 emissions between dates in the *Sesarma* treatments increased by 31 July 2014. We attribute this increase to the total consumption of *S. patens* by *Sesarma* and subsequent respiration by the remaining plant roots and soils. *Uca* did not have a significant impact in the low inundation treatments compared to the controls, however it did significantly reduce the CO_2 uptake in the high inundation treatment, which was unexpected. Methane (CH_4) fluxes were negligible on 2 July 2014 (not shown) and were variable and not significantly affected by treatments on 31 July 2014 (Figure 4, Table 2).

Soil pH was significantly higher in the high elevation treatments on 2 July 2014, but crabs did not have a significant affect (Table 3-4). No significant difference in soil pH was shown in any of the treatments for 31 July 2014. High elevation had significantly higher soil redox (Figure 5) and moisture content (Figure 6) on both dates while crabs had no significant effect on either variable (Table 2-3). Average soil salinities ranged between 28-55 ppt but did not significantly differ between treatments. There were no strong significant relationships between edaphic parameters and GHG fluxes.

There was a surprising significantly lower emission of CO_2 in the dieback zone compared to the control, which does not support our hypothesis (Figure 6A, Table 5). There was a significant effect of date such that there was consumption of CO_2 by the

late fall compared to the early fall collection dates (Figure 6A). There was no significant difference in CH_4 fluxes between treatments or date of collection though there was also a trend of lower fluxes for the late fall sample date (Figure 6B, Table 5). N_2O was negligible in all treatments during all sample dates.

Redox potential, soil moisture and temperature were significantly different between treatments, date, and the combination of treatment x date (Table 5-6). There was a significant negative relationship between moisture and CO_2 (R^2=0.45, p=0.01) driven by lower moisture levels in the control plots, likely due to greater uptake by the salt marsh grasses compared to the dieback zone. There was a positive relationship between pH and CH_4 (R^2=0.50, p<0.01) driven by higher pH levels in the controls plots.

These data were collected during the fall season where microbial activity tend to be lower due to lower temperatures (Table 6), thus the lower CO_2 in the dieback region is likely due to lower microbial activity compared to high respiration by the plants, which is common for the fall months. Future research should focus on the growing season, which is the time salt marshes grasses and associated microbial communities are more active.

TABLES AND FIGURES

Table 1. Average results of edaphic parameters for mangrove study ± standard error.

Site	pH	Redox (mV)	Soil moisture (%)	Salinity (ppt)
Red mangrove sediment experiment				
Cayos (pristine)	6.8 ± 0.08	-90.5 ± 20.0	65.2 ± 2.5	37.0 ± 0.6
Mar Negro (anthropogenic)	6.7 ± 0.08	-72.7 ± 16.1	65.6 ± 1.1	31.5 ±2.5
Anthropogenic red mangrove response to N additions experiment				
Control	6.84 ±0.12	-119 ± 44.0	64.7 ± 0.3	41 ± 1.3
Nutrient	6.79 ±0.16	-39 ± 74. 3	64.2 ± 1.2	42 ± 3.9

Table 2. Results of a two factor ANOVA for mangrove study without N additions. *statistical significance at $\alpha = 0.05$

Gas/Date	F-ratio	p-value whole model	p-value crab	p-value inundation	p-value crab x inundation
CO_2 - 2014 July 02	$F_{5,12}$=4.92	0.01*	0.06	0.01*	0.06
CO_2 - 2014 July 31	$F_{5,12}$=5.11	0.01*	<0.01*	0.77	0.04*
CH_4 - 2014 July 31	$F_{5,12}$=0.26	0.93	0.79	0.67	0.74

Table 3. Average edaphic parameter results for 2 and 31 July 2014 for crab and sea level rise study ± standard error.

Crab	Inundation	pH	Redox (mV)	Moisture (%)	Salinity (ppt)
2-Jul-2014					
None (Control)	Low	7.7 ± 0.1	394.3 ± 15.2	55.5 ± 1.4	55.3 ± 17.6
None (Control)	High	7.6 ± 0.1	34.3 ± 54.0	56.7 ± 1.0	34.0 ± 0
Sesarma	Low	7.7 ± 0.1	364.3 ± 22.7	50.7 ± 0.7	41.7 ± 4.5
Sesarma	High	7.5 ± 0.1	72.7 ± 100.3	57.3 ± 0.8	33.0 ± 1.0
Uca	Low	7.7 ± 0.1	425.3 ± 43.1	53.3 ± 2.0	32.7 ± 7.7
Uca	High	7.5 ± 0.1	186.0 ± 39.7	56.6 ± 0.5	32.7 ± 0.9
31-Jul-2014					
None (Control)	Low	7.7 ± 0.1	406.3 ± 22.0	54.4 ± 2.8	38.7 ± 5.7
None (Control)	High	7.6 ± 0.1	-24.7 ± 21.7	61.4 ± 1.5	32.7 ± 0.7
Sesarma	Low	7.7 ± 0.1	377.0 ± 17.6	54.0 ± 2.3	44.2 ± 17.3
Sesarma	High	7.8 ± 0.1	-44.7 ± 14.2	63.2 ± 0.6	33.0 ± 0.6
Uca	Low	7.8 ± 0.1	382.3 ± 3.5	52.6 ± 1.6	28.7 ± 8.7
Uca	High	7.7 ± 0.1	23.3 ± 20.2	60.2 ± 2.3	32.3 ± 1.2

Table 4. Two-factor ANOVA results for edaphic parameters. *statistical significance at $\alpha = 0.05$

Parameter	F-ratio	p-value whole model	p-value crab	p-value inundation	p-value crab x inundation
2-Jul-2014					
pH	$F_{5,12}=1.77$	0.2	0.74	0.02*	0.89
Redox	$F_{5,12}=10.24$	<0.001*	0.2	<0.0001*	0.54
Moisture	$F_{5,12}=5.12$	0.01*	0.2	<0.01*	0.08
Salinity	$F_{5,12}=1.27$	0.34	0.36	0.15	0.44
31-Jul-2014					
pH	$F_{5,12}=0.59$	0.71	0.7	0.93	0.36
Redox	$F_{5,12}=157.84$	<0.0001*	0.15	<0.0001*	0.13
Moisture	$F_{5,12}=5.18$	<0.01*	0.55	<0.001*	0.84
Salinity	$F_{5,12}=0.45$	0.7	0.62	0.52	0.67

Table 8. Two factor ANOVA results for in situ salt marsh dieback study. *statistical significance at $\alpha = 0.05$

Parameter	F-ratio	p-value whole model	p-value treatment	p-value time	p-value treatment x time
Greenhouse gases					
CO_2	$F_{3,12}=3.46$	0.05*	0.05*	0.03*	0.54
CH_4	$F_{3,12}=1.23$	0.35	0.09	0.56	0.66
Edaphic parameters					
pH	$F_{3,12}=5.03$	0.02*	0.95	0.002*	0.49
Redox	$F_{3,12}=48.69$	<0.0001*	<0.0001*	<0.0001*	<0.0001*
Moisture	$F_{3,12}=16.00$	<0.001*	<0.0001*	0.02*	0.01*
Salinity	$F_{3,12}=12.46$	<0.001*	0.24	0.001	0.41
Temperature	$F_{3,12}=523.31$	<0.001*	<0.001*	<0.001*	<0.001*

Table 6. Edaphic parameter results for *in situ* salt marsh dieback study ± standard error.

Plot	Redox (mV)	pH	moisture (%)	Salinity (ppt)	Temperature (°C)
Early Fall 2015					
Intact	-195.3 ± 28.8	7.0 ± 0.1	45.2 ± 2.3	33.0 ± 1.4	12.9 ± 0.2
Dieback	-256.0 ± 49.7	7.1 ± 0.2	63.6 ± 0.7	37.0 ± 3.2	12.8 ± 0.2
Late Fall 2015					
Intact	231.3 ± 5.7	6.6 ± 0.1	57.4 ± 2.7	23.3 ± 1.5	5.1 ± 0.2
Dieback	-242.3 ± 33.4	6.5 ± 0.2	63.3 ± 2.3	24.0 ± 0.7	6.8 ± 0.2

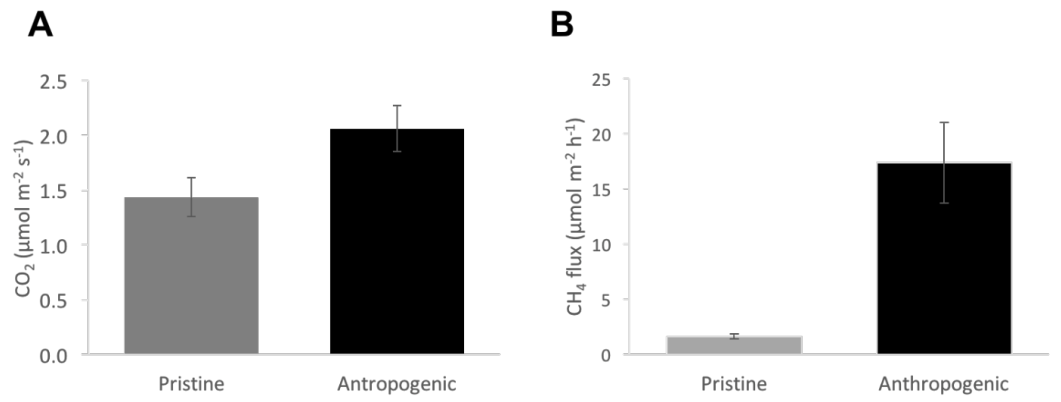

Figure 1. Greenhouse gas fluxes for pristine and anthropogenic mangrove sediments. A) CO_2 flux; B) CH_4 flux. Standard error bars shown.

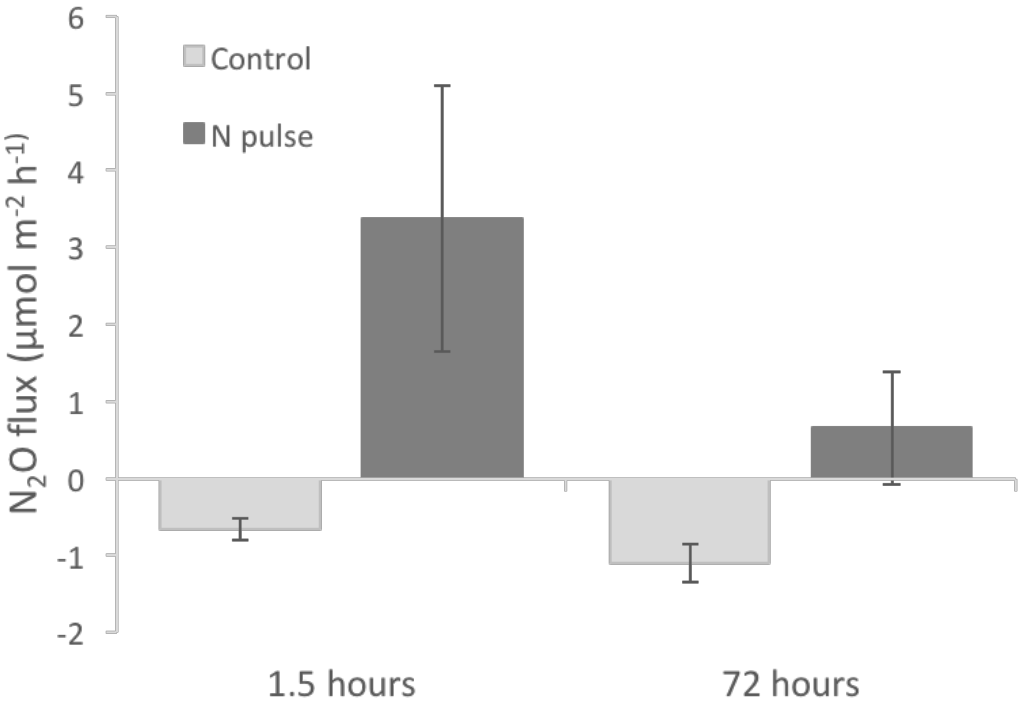

Figure 2. N_2O flux for N pulse experiment in the anthropogenically influenced site. Standard error bars shown.

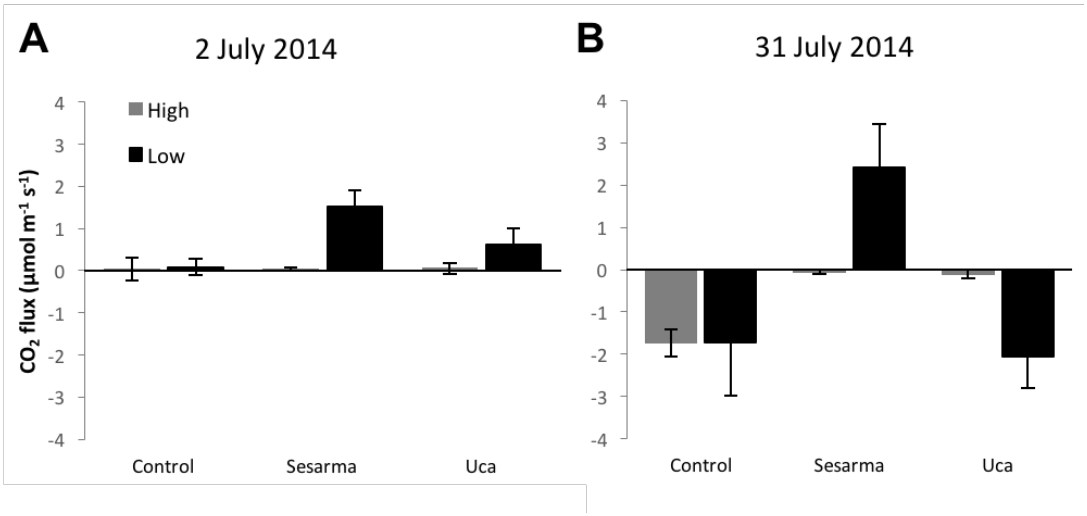

Figure 3. CO_2 fluxes for crab and sea level rise study for A) 2 July 2014 and B) 31 July 2014. High= High inundation, Low= Low inundation. Standard error bars shown.

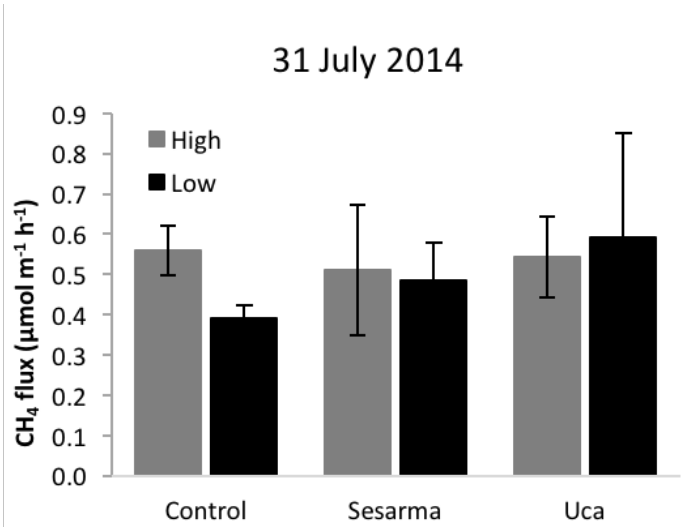

Figure 4. CH_4 fluxes for crab and sea level rise study for 31 July 2014. High= High inundation, Low= Low inundation. Standard error bars shown.

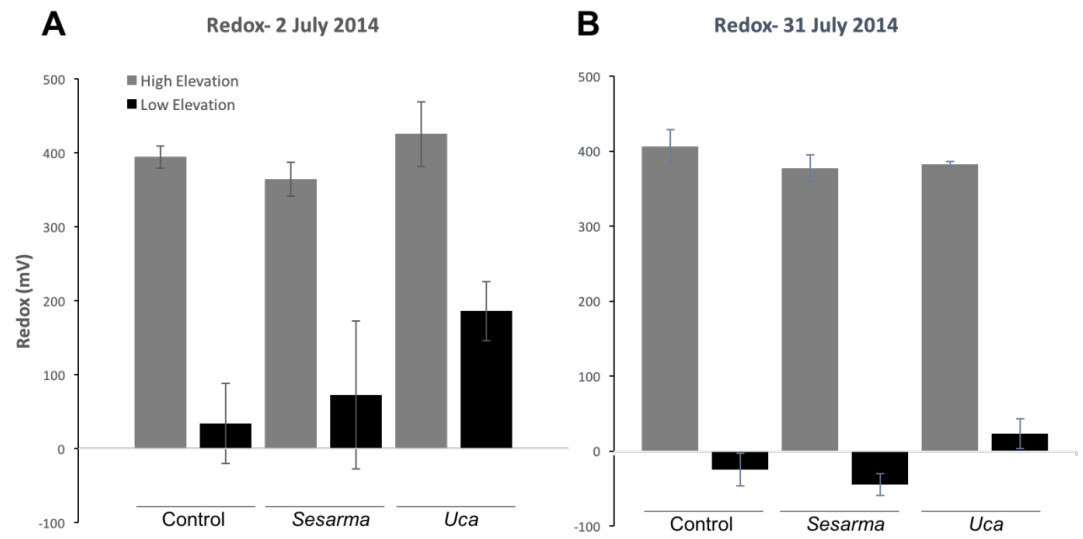

Figure 5. Average soil redox levels for crab and sea level rise study A) 2 July 2014 and b) 31 July 2014. Standard error bars shown.

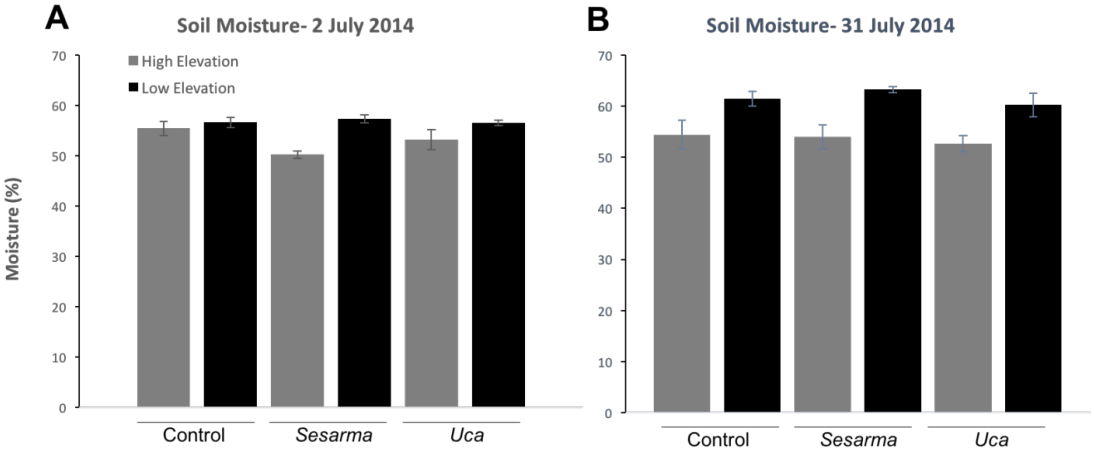

Figure 6. Soil moisture content for A) 2 July 2014 and B) 31 July 2014. Standard error bars shown.

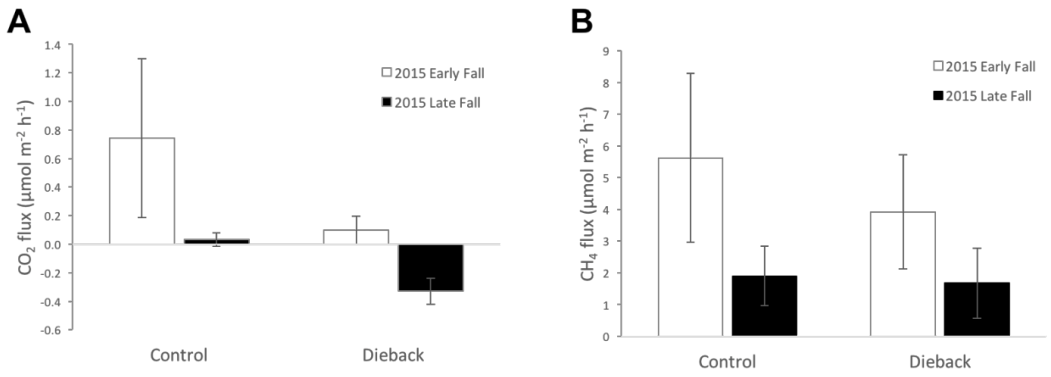

Figure 7. GHG fluxes for in situ dieback experiment. A) CO_2 fluxes, B) CH_4 fluxes. Standard error bars shown.

BIBLIOGRAPHY

Alber M, Swenson EM, Adamowicz SC, Mendelssohn IA. 2008. Salt Marsh Dieback: An overview of recent events in the US. Estuar. Coast. Shelf Sci. 80:1–11.

Altieri AH, Bertness MD, Coverdale TC, Herrmann NC, Angelini C. 2012. A trophic cascade triggers collapse of a salt-marsh ecosystem with intensive recreational fishing. Ecology 93:1402–1410.

Altieri AH, Gedan KB. 2015. Climate change and dead zones. Glob. Change Biol. 21:1395–1406.

Bertness MD, Holdredge C, Altieri AH. 2009. Substrate mediates consumer control of salt marsh cordgrass on Cape Cod, New England. Ecology 90:2108–2117.

Boulêtreau S, Salvo E, Lyautey E, Mastrorillo S, Garabetian F. 2012. Temperature dependence of denitrification in phototrophic river biofilms. Sci. Total Environ. 416:323–328.

Bowen JL, Valiela I. 2008 May 1. Using δ15N to assess coupling between watersheds and estuaries in temperate and tropical regions. J. Coast. Res.:804–813.

Call M, Maher DT, Santos IR, Ruiz-Halpern S, Mangion P, Sanders CJ, Erler DV, Oakes JM, Rosentreter J, Murray R, et al. 2015. Spatial and temporal variability of carbon dioxide and methane fluxes over semi-diurnal and spring–neap–spring timescales in a mangrove creek. Geochim. Cosmochim. Acta 150:211–225.

Canion A, Kostka JE, Gihring TM, Huettel M, van Beusekom JEE, Gao H, Lavik G, Kuypers MMM. 2014. Temperature response of denitrification and anammox reveals the adaptation of microbial communities to in situ temperatures in permeable marine sediments that span 50 degrees in latitude. Biogeosciences 11:309–320.

Carmichael RH, Shriver AC, Valiela I. 2012. Bivalve response to estuarine eutrophication: The balance between enhanced food supply and habitat alterations. J. Shellfish Res. 31:1–11.

Caughlan J, Ansell AD. 1964. A direct method for determining the pumping rate of siphons bivalve. J. cons. int. Explor. Mer. 29:205–213.

Chauhan R, Ramanathan A, Adhya TK. 2008. Assessment of methane and nitrous oxide flux from mangroves along Eastern coast of India. Geofluids 8:321–332.

Chen GC, Tam NFY, Ye Y. 2012. Spatial and seasonal variations of atmospheric N2O and CO2 fluxes from a subtropical mangrove swamp and their relationships with soil characteristics. Soil Biol. Biochem. 48:175–181.

Chmura GL, Anisfeld SC, Cahoon DR, Lynch JC. 2003. Global carbon sequestration in tidal, saline wetland soils. Glob. Biogeochem. Cycles 17:1111.

Codiga DL, Stoffel HE, Deacutis CF, Kiernan S, Oviatt CA. 2009. Narragansett Bay hypoxic event characteristics based on fixed-site monitoring network time series: intermittency, geographic distribution, spatial synchronicity, and interannual variability. Estuaries Coasts 32:621–641.

Corredor JE, Morell JM, Bauza J. 1999. Atmospheric nitrous oxide fluxes from mangrove sediments. Mar. Pollut. Bull. 38:473–478.

Craft C, Clough J, Ehman J, Joye S, Park R, Pennings S, Guo H, Machmuller M. 2009. Forecasting the effects of accelerated sea-level rise on tidal marsh ecosystem services. Front. Ecol. Environ. 7:73–78.

Deacutis CF. 2008. Evidence of ecological impacts from excess nutrients in upper Narragansett Bay. In: Desbonnet A, Costa-Pierce BA, editors. Science for Ecosystem-based Management. Springer New York. (Springer Series on Environmental Management). p. 349–381.

Deacutis CF, Murray D, Prell W, Saarman E, Korhun L. 2006. Hypoxia in the upper half of Narragansett Bay, RI, during August 2001 and 2002. Northeast. Nat. 13:173–198.

Dickinson GH, Ivanina AV, Matoo OB, Pörtner HO, Lannig G, Bock C, Beniash E, Sokolova IM. 2012. Interactive effects of salinity and elevated CO2 levels on juvenile eastern oysters, Crassostrea virginica. J. Exp. Biol. 215:29–43.

Donato DC, Kauffman JB, Murdiyarso D, Kurnianto S, Stidham M, Kanninen M. 2011. Mangroves among the most carbon-rich forests in the tropics. Nat. Geosci. 4:293–297.

Dove MC, Sammut J. 2007. Impacts of estuarine acidification on survival and growth of sydney rock oysters, Saccostrea glomerata (Gould 1850). J. Shellfish Res. 26:519–527.

Duarte CM, Hendriks IE, Moore TS, Olsen YS, Steckbauer A, Ramajo L, Carstensen J, Trotter JA, McCulloch M. 2013. Is Ocean Acidification an Open-Ocean Syndrome? Understanding Anthropogenic Impacts on Seawater pH. Estuaries Coasts 36:221–236.

Edwards D, Hurley D, Wenner E. 2004 Sep 1. Nonparametric Harmonic Analysis of Estuarine Water-Quality Data: A National Estuarine Research Reserve Case Study. J. Coast. Res.:75–92.

Figueiredo-Barros MP, Caliman A, Leal JJF, Bozelli RL, Farjalla VF, Esteves FA. 2009. Benthic bioturbator enhances CH4 fluxes among aquatic compartments and atmosphere in experimental microcosms. Can. J. Fish. Aquat. Sci. 66:1649–1657.

Forster P, Ramaswamy P, Artaxo T, Berntsen R, Betts D., Fahey J. 2007. Changes in atmospheric constituents and in radiative forcing. In: Climate Change 2007: The

Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change.

Goreau TJ, Kaplan WA, Wofsy SC, McElroy MB, Valois FW, Watson SW. 1980. Production of NO2- and N2O by Nitrifying Bacteria at Reduced Concentrations of Oxygen. Appl. Environ. Microbiol. 40:526–532.

Gruber N, Galloway JN. 2008. An Earth-system perspective of the global nitrogen cycle. Nature 451:293–296.

Heisterkamp IM, Schramm A, Beer D de, Stief P. 2010. Nitrous oxide production associated with coastal marine invertebrates. Mar. Ecol. Prog. Ser. 415:1–9.

Heisterkamp IM, Schramm A, Larsen LH, Svenningsen NB, Lavik G, de Beer D, Stief P. 2013. Shell biofilm-associated nitrous oxide production in marine molluscs: processes, precursors and relative importance. Environ. Microbiol. 15:1943–1955.

Hiebenthal C, Philipp EER, Eisenhauer A, Wahl M. 2012. Effects of seawater pCO2 and temperature on shell growth, shell stability, condition and cellular stress of Western Baltic Sea Mytilus edulis (L.) and Arctica islandica (L.). Mar. Biol. 160:2073–2087.

Holdredge C, Bertness MD, Altieri AH. 2009. Role of crab herbivory in die-off of New England salt marshes. Conserv. Biol. 23:672–679.

Jorgensen BB, Sorensen J. 1985. Seasonal cycles of O, NO; and SO:-reduction in estuarine sediments: the significance of an NO; reduction maximum in spring. Mar. Ecol. Prog. Ser. 24:65–74.

Kellogg ML, Smyth AR, Luckenbach MW, Carmichael RH, Brown BL, Cornwell JC, Piehler MF, Owens MS, Dalrymple DJ, Higgins CB. 2014. Use of oysters to mitigate eutrophication in coastal waters. Estuar. Coast. Shelf Sci. 151:156–168.

Kittner HR. 2005. Effect of temperature on filtration rate in the mussel Mytilus edulis: No evidence for temperature compensation. Mar. Ecol.-Prog. Ser. 305:147–152.

Kremer J, Nixon SW. 1979. A coastal marine ecosystem. Simulation and analysis. Limnol. Oceanogr. 24:598–598.

Kroeze C, Seitzinger SP. 1998. Nitrogen inputs to rivers, estuaries and continental shelves and related nitrous oxide emissions in 1990 and 2050: a global model. Nutr. Cycl. Agroecosystems 52:195–212.

Lannig G, Flores JF, Sokolova IM. 2006. Temperature-dependent stress response in oysters, Crassostrea virginica: Pollution reduces temperature tolerance in oysters. Aquat. Toxicol. 79:278–287.

Mackenzie CL, Lynch SA, Culloty SC, Malham SK. 2014. Future oceanic warming and acidification alter immune response and disease status in a commercial shellfish species, Mytilus edulis L. PLOS ONE 9:e99712.

Matoo OB, Ivanina AV, Ullstad C, Beniash E, Sokolova IM. 2013. Interactive effects of elevated temperature and CO2 levels on metabolism and oxidative stress in two common marine bivalves (Crassostrea virginica and Mercenaria mercenaria). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 164:545–553.

McHenery JG, Birkbeck TH. 1985. Uptake and processing of cultured microorganisms by bivalves. J. Exp. Mar. Biol. Ecol. 90:145–163.

Mora C, Wei C-L, Rollo A, Amaro T, Baco AR, Billett D, Bopp L, Chen Q, Collier M, Danovaro R, et al. 2013. Biotic and human vulnerability to projected changes in ocean biogeochemistry over the 21st century. Mace GM, editor. PLoS Biol. 11:e1001682.

Moseman-Valtierra S, Kroeger KD, Crusius J, Baldwin S, Green A, Brooks TW, Pugh E. 2015. Substantial nitrous oxide emissions from intertidal sediments and groundwater in anthropogenically-impacted West Falmouth Harbor, Massachusetts. Chemosphere 119:1281–1288.

Muñoz-Hincapié M, Morell JM, Corredor JE. 2002. Increase of nitrous oxide flux to the atmosphere upon nitrogen addition to red mangroves sediments. Mar. Pollut. Bull. 44:992–996.

Nixon SW, Fulweiler RW, Buckley BA, Granger SL, Nowicki BL, Henry KM. 2009. The impact of changing climate on phenology, productivity, and benthic–pelagic coupling in Narragansett Bay. Estuar. Coast. Shelf Sci. 82:1–18.

Oczkowski A, Nixon S, Henry K, DiMilla P, Pilson M, Granger S, Buckley B, Thornber C, McKinney R, Chaves J. 2008. Distribution and trophic importance of anthropogenic nitrogen in Narragansett Bay: an assessment using stable isotopes. Estuaries Coasts 31:53–69.

Oczkowski A, Wigand C, Hanson A, Markham E, Miller KM, Johnson R. 2015. Nitrogen Retention in salt marsh systems across nutrient-enrichment, elevation, and erecipitation regimes: a multiple-stressor experiment. Estuaries Coasts 39:68–81.

Oviatt C, Keller A, Reed L. 2002. Annual primary production in Narragansett Bay with no bay-wide winter–spring phytoplankton bloom. Estuar. Coast. Shelf Sci. 54:1013–1026.

Piehler MF, Smyth AR. 2011. Habitat-specific distinctions in estuarine denitrification affect both ecosystem function and services. Ecosphere 2:1–17.

Pilson MEQ. 1985. On the residence time of water in Narragansett Bay. Estuaries 8:2–14.

Riisgård H. 1988. Efficiency of particle retention and filtration rate in 6 species of Northeast American bivalves. Mar. Ecol. Prog. Ser. 45:217–223.

Riisgard HU, Randløv A. 1981. Energy budgest, growth and filtration rates in Mytilus edulis at different algal concentrations. Mar. Biol. 61:227–234.

Rivera-Monroy VH, Twilley RR. 1996. The relative role of denitrification and immobilization in the fate of inorganic nitrogen in mangrove sediments (Terminos Lagoon, Mexico). Limnol. Oceanogr. 41:284–296.

Schulte DM, Burke RP, Lipcius RN. 2009. Unprecedented Restoration of a Native Oyster Metapopulation. Science 325:1124–1128.

Seitzinger SP, Pilson MEQ, Nixon SW. 1983. Nitrous oxide production in nearshore marine sediments. Science 222:1244–1246.

Smyth A, Geraldi N, Piehler M. 2013. Oyster-mediated benthic-pelagic coupling modifies nitrogen pools and processes. Mar. Ecol. Prog. Ser. 493:23–30.

Stief P. 2013. Stimulation of microbial nitrogen cycling in aquatic ecosystems by benthic macrofauna: mechanisms and environmental implications. Biogeosciences 10:7829–7846.

Stief P, Eller G. 2006. The gut microenvironment of sediment-dwelling Chironomus plumosus larvae as characterised with O2, pH, and redox microsensors. J. Comp. Physiol. B 176:673–683.

Stief P, Poulsen M, Nielsen LP, Brix H, Schramm A. 2009. Nitrous oxide emission by aquatic macrofauna. Proc. Natl. Acad. Sci. 106:4296–4300.

Stief P, Schramm A. 2010. Regulation of nitrous oxide emission associated with benthic invertebrates. Freshw. Biol.

Sundbäck K, Miles A, Goeransson E. 2000. Nitrogen fluxes, denitrification and the role of microphytobenthos in microtidal shallow-water sediments: an annual study. Mar. Ecol. Prog. Ser. 200:59–76.

Svenningsen NB, Heisterkamp IM, Sigby-Clausen M, Larsen LH, Nielsen LP, Stief P, Schramm A. 2012. Shell biofilm nitrification and gut denitrification contribute to emission of nitrous oxide by the invasive freshwater mussel Dreissena polymorpha (zebra mussel). Appl. Environ. Microbiol. 78:4505–4509.

Tam JC, Scrosati RA. 2011. Mussel and dogwhelk distribution along the north-west Atlantic coast: testing predictions derived from the abundant-centre model. J. Biogeogr. 38:1536–1545.

Thelen BA, Thiet RK. 2009. Molluscan community recovery following partial tidal restoration of a New England estuary, U.S.A. Restor. Ecol. 17:695–703.

Vieillard AM, Fulweiler RW. 2014. Tidal pulsing alters nitrous oxide fluxes in a temperate intertidal mudflat. Ecology 95:1960–1971.

Walter S, Peeken I, Lochte K, Webb A, Bange HW. 2005. Nitrous oxide measurements during EIFEX, the European Iron Fertilization Experiment in the subpolar South Atlantic Ocean. Geophys. Res. Lett. 32:L23613.

Weiss RF, Price BA. 1980. Nitrous oxide solubility in water and seawater. Mar. Chem. 8:347–359.

Wrage N, Velthof GL, van Beusichem ML, Oenema O. 2001. Role of nitrifier denitrification in the production of nitrous oxide. Soil Biol. Biochem. 33:1723–1732.

Zumft WG. 1997. Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. 61:533–616.